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LIFE HISTORY OF THE HAIRY-ROOT ORGANISM IN RELATION TO ITS PATHOGENESIS ON NURSERY APPLE TREES [1]

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INTRODUCTION

In studies of infectious hairy root carried on during the last 4 years, special consideration has been given to the life history of the causal organism in relation to its pathogenesis on nursery apple trees. This disease has been so prevalent on grafted apple trees in the nursery that it has become of considerable economic importance. The causal organism, *Phytomonas rhizogenes* Riker et al., has recently been differentiated by Riker and his associates (*26*)[3] as a new species distinct from that causing crown gall, *P. tumefaciens* (Smith and Town.) Bergey et al. Previously it had been considered an apple strain of the crown-gall organism by Smith et al. (*36*) and more completely developed as such by Siegler (*33, 34*).

The name "hairy root" appears to have been first introduced into literature by Stewart, Rolfs, and Hall (*37*). Following the earlier work of Hedgcock (*11*) on the identity of the complex of malformations occurring on apple, a number of well-known papers appeared. Recently a number of diseases have been separated from this complex on the basis of cause, viz, (1) infectious hairy root, (2) crown gall, (3) wound overgrowth, and (4) nonparasitic hairy root. Of these, infectious hairy root is now perhaps the most important from the economic standpoint.

The host range of the hairy-root organism is little understood. Up to the present time the writer has found it reported under natural conditions only on apple. However, cross-inoculation studies by various workers, including Smith et al. (*36*), Riker et al. (*25*), Brown (*6*), Banfield (*3*), and Riker et al. (*26*), have demonstrated the pathogenicity of these bacteria on such plants as sugar beet (*Beta vulgaris* L.) quince (*Cydonia oblonga* Mill.), rose (*Rosa setigera* Michx.), honeysuckle (*Lonicera morrowi* Gray), Paris daisy (*Chrysanthemum frutescens* L.), balsam (*Impatiens balsamina* L.), bryophyllum (*Bryophyllum pinnatum* Kurz), red raspberry (*Rubus idaeus* L.), bean (*Phaseolus vulgaris* L.), and sedum (*Sedum spectabile* Bor.).

Hairy root appears to be widely distributed. Hedgcock (*11*), who reported crown gall from all the States of the United States except

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[3] Reference is made by number (italic) to Literature Cited, p. 883.

Nevada, stated that "the forms of the disease known as hairy root have been found as widely disseminated as crown gall on apple trees in nurseries and orchards in the United States." He also reported forms of hairy root from Germany, Netherlands, France, Canada, and New Zealand. Doidge (*8*) recorded the presence of the disease in South Africa, and Noble (*18*) in Australia. The disease seems widely disseminated, but the difficulties of diagnosis raise a question as to the accuracy of some of the reports.

The economic importance of infectious hairy root, although considerable, is hard to estimate since no information is available as to how much of the loss due to malformations may be attributed to this disease. Studies in which the writer has participated for more than 4 years, in nurseries from Wisconsin to Oklahoma, reveal that while other difficulties in this complex have been largely eliminated hairy root still remains a factor of considerable importance in certain places.

Control measures at the present time are only partially satisfactory. Von Schrenk and Hedgcock (*39*) laid the foundation for the most successful of later attempts to control the various malformations at the unions of piece-root grafts when they noted that these enlargements usually appeared at the graft union, that using root and scion pieces of nearly the same diameter reduced overgrowths, and that wrapping the unions with certain materials such as cloth and rubber considerably increased the percentage of smooth trees over those wrapped with other materials. Since the publication of their report many control measures have been suggested by different workers, as, for example, Melhus and Maney (*15*), Wormald and Grubb (*41*), Riker and Keitt (*30*), Waite and Siegler (*40*), Melhus, Muncie, and Fisk (*16*), and Maney and Pickett (*14*). Additional studies by Riker and his associates (*21, 24, 27, 31*) have repeatedly demonstrated the value of nurseryman's tape, a special kind of adhesive plaster. Since the discovery that this tape prevents a large percentage of union malformations without producing any ill effects, it has come into common use. Among the nurserymen there is a feeling that the saving in handling the grafts bound with this wrapper more than pays for its extra cost. In addition, there is a considerable increase in the number of clean trees. The use of nurseryman's tape has eliminated on an average more than half of the various outgrowths at the union. Those that remain are mostly hairy root.

The persistence of malformations, chiefly of the hairy-root type, caused by *Phytomonas rhizogenes*, appeared to warrant a study of the life history of the causal organism in relation to pathogenesis. It was hoped that such a study would not only increase the available information on the fundamental activity of this organism but would also define critical points at which the application of control measures might be more effective.

IDENTITY OF HAIRY ROOT

Before studying the pathogenic life history of infectious hairy root it seemed desirable to make a reexamination of the identity of hairy root as contrasted with crown gall, wound overgrowth, and other malformations occurring on the underground parts of nursery apple trees.

The isolation of the hairy-root organism was attempted from a variety of malformations on Wealthy apple trees collected at random

at digging time from the experimental plots at Madison, Wis., and Topeka, Kans. It was found from the outward appearance and interior structure that these overgrowths could be roughly classified into three groups: (1) Convoluted, with roots, characterized by a hard vascular interior and a soft exterior layer of variable thickness that turned brown rapidly when exposed to the air; (2) convoluted, without roots, identical with the foregoing except for the absence of roots; and (3) undulated, without roots and, unlike the other enlargements, chiefly made up of whitish vascular cortex. The enlargements differed considerably in appearance and in the number of emerging roots, making them difficult to classify. Nevertheless it seemed important to attempt isolations from such specimens.

The method of isolation was as follows: From a specimen of each type of overgrowth several cubes of overgrowth tissue, approximately 3 mm on a side, were removed from the soft tissue, if present, under aseptic conditions and dropped into three Petri dishes each containing 1 cc of sterile distilled water. A sterilized scalpel was used to macerate the tissue in the water. After an interval of about 10 minutes dilutions were made from each plate to three successive Petri dishes. Patel's (*19*) bile agar was then added. The poured plates were incubated at 28° C. and examined after 1 week. The identity of the bacteria that appeared was determined after inoculations on sedum and apple and after cultural examination, as suggested by A. J. Riker, on sodium selenite agar. A summary of these studies is given in table 1, showing that a majority of the convoluted enlargements with roots were infectious hairy root and that a considerable part of the convoluted enlargements without roots were also hairy root. The undulated enlargements did not yield the infectious agent and in all probability they were nonparasitic overgrowths. Since the experiment described below showed the isolation technic to be reasonably accurate, it appears that many of the specimens contained tissue not primarily stimulated by the hairy-root bacteria. Perhaps the reaction induced by the hairy-root tissue may have stimulated nonparasitic growth in some cases. These results appear to be in conformity with those of Riker et al. (*26*).

TABLE 1.—*Summary of isolation trials from different kinds of enlargements*

Surface character of enlargement [a]	Isolation trials	Enlargements yielding—		
		Phytomonas rhizogenes	Nonpathogenic bacteria	No bacteria
	Number	*Percent*	*Percent*	*Percent*
Convoluted, with roots	114	60	24	16
Convoluted, without roots	13	39	54	7
Undulated, without roots	25	0	32	68

[a] Characterization of these enlargements is given in the text.

The hairy-root organism was reisolated from hairy-root enlargements induced on nursery apple trees by inoculating the stems below ground. These induced enlargements, ranging in age from 0 to 24 weeks, covered the complete range of symptoms. The same isolation technic was employed as in previous experiments. The results are summarized

in table 2. All stages of the disease yielded the causal organism. Out of 129 trials, the hairy-root organism was recovered in 87 percent, indicating that the causal organism is usually associated with the symptoms of the disease. Nonpathogenic bacteria were recovered in 13 percent of the trials. None of the enlargements was found to be free from bacteria.

TABLE 2.—*Summary of reisolation trials from infectious hairy-root enlargements of different ages, induced by inoculations on nursery apple trees*

Year	Period following inoculations	Isolation trials	Enlargements yielding—	
			Phytomonas rhizogenes	Nonpathogenic bacteria
	Weeks	*Number*	*Percent*	*Percent*
1929	0–4	27	93	7
	5–8	20	60	40
	9–12	8	50	50
1930	0–4	18	100	0
	5–8	14	80	20
	9–12	10	100	0
	13–16	14	100	0
	17–20	12	100	0
	21–24	6	100	0
Total		129	87	13

The roots of infectious hairy root have not been found to contain the hairy-root bacteria. Only negative results were secured from attempts to isolate the organism from the tissues of 37 fleshy hairy roots more than one fourth of an inch long, which had emerged from the basal enlargements. This finding confirms the work of Smith et al. (*36*) and Riker et al. (*26*), who found the bacteria to be present only in the basal enlargements.

Isolation trials from crown gall and wound overgrowth induced by inoculations and wire girdles, respectively, appear to establish the identity of these malformations. A series of reisolation studies somewhat less extensive than those conducted on hairy root were made on induced crown gall and wound overgrowth. In 26 trials the crown-gall organism was found constantly associated with the crown-gall symptoms in all stages from 0 to 12 weeks. As no pathogenic bacteria were secured in the 10 trials from wound overgrowths, these malformations apparently are distinct from each other and from hairy root. These results are in accord with those of Riker and Keitt (*30*), Muncie (*17*), Siegler (*33*), Brown (*6*), and others, and, in the writer's opinion, establish the identity of the hairy-root disease.

ENTRANCE OF ORGANISM INTO HOST

MATERIALS AND METHODS

In most of the field studies the Yellow Transparent variety of apple was selected as host plant because it was considered relatively susceptible to infectious hairy root and because it was grown in sufficient numbers for the studies projected. Both first- and second-year trees were utilized. These trees were produced from piece-root grafts made from scions and roots grown in Kansas.

The principal plants used in the greenhouse were Paris daisy, bean, sugar beet, sedum, and apple. In a series of pathogenicity studies

sedum and sugar beet were found to be the best plants tried for greenhouse studies. In these studies an average of 54 inoculations were made for each kind of plant. The following percentages of wounds in the respective hosts showed hairy-root symptoms 2 months after inoculation: Balsam, 36; bean, 58; bryophyllum, 57; Paris daisy (single yellow), 66; Paris daisy (single white), 36; sedum, 100; sugar beet, 100; begonia, 0; and tomato, 0. A similar study of 240 wound inoculations on the Fameuse variety of apple grown in pots showed 52 percent infected.

The bacterial culture chiefly employed was the progeny of a single cell, strain C–1 (fig. 1), of the hairy-root organism, a detailed description of which is given by Wright, Hendrickson, and Riker (*42*). At 3-month intervals this strain was checked for comparative pathogenicity against four sister single-cell strains, C–10, C–11, C–12, and C–13. Three-day-old transfers grown on potato-mannite agar gave abundant growth and were used for inoculation.

The method of inoculation on herbaceous plants was by needle puncture. The method of inoculation on apple grafts, unless otherwise noted, was as follows: On one side of the row a trench was made in the soil to the depth of the union, about 3 inches away from the trees to avoid injuring them. By means of a dibble the soil was removed from around the individual trees, and the stems were wiped free from soil. Drops of a subculture of the bacteria were then applied with a cotton swab to the stem surface, usually in five places spaced about 1 inch apart. With a scalpel, held at an angle, two thrusts were made through the drops of culture deep into the stem. During the 1930 season, strips of adhesive tape were applied over the wounds to reduce chance contamination of the controls from the soil and to keep out insects. Promptly after inoculation the soil was thrown back into the trench and was hilled up several inches so as to bring it about 2 inches above the topmost wound.

WOUNDS AS INFECTION COURTS

The entrance of bacteria into the host has appeared to be a critical stage in the life history of the hairy-root organism; consequently a series of studies was undertaken to clarify this point.

As the bacteria seemed to enter the host plant only through wounds, the necessity for wounds as points of infection was tested in the following preliminary experiments. The hairy-root organism was washed over the surface of 20 Paris daisy plants. Ten of these were wounded with needle punctures in five places each, and the others were held without injury as controls. After 2 months of incubation all the unwounded plants were free from disease, whereas 41 out of 50, or 82 percent of the places wounded, showed hairy-root symptoms. Two repetitions of this experiment gave similar results. An experiment on sugar beets, in which the same number of plants were used and the same number of wounds were made, resulted in the unwounded plants remaining healthy and all 50, or 100 percent, of the wounded plants becoming infected. In two similar experiments on bean, 62 and 34 percent, respectively, of the places wounded became infected, but none of the unwounded plants showed symptoms of disease. In an experiment on the Yellow Transparent variety of apple the bacteria were washed over 20 stems below ground. Ten of these were wounded in five places each with scalpel cuts. Two months later

the unwounded stems were healthy and 28 of the 50 places wounded, or 56 percent, showed symptoms of the disease. These results are in accord with those obtained by Smith et al. (*36*), Riker (*21*), and Muncie (*17*), in work with the crown-gall organism. From these studies it appeared that infection occurred only through wounds. Attention was next directed to the various kinds of wounds that might be encountered.

Infection followed the introduction of the bacteria into the tissue. This was determined by several tests. Drops of a fresh culture of the hairy-root organism from a cotton swab were placed in five different places on the stems of 10 Paris daisy plants. The bacteria were carried into the tissues by thrusting a needle through the bacterial masses and then entirely through the stems several times. After 2 months of incubation, 38 of the 50 places wounded, or 76 percent, showed the disease symptoms. A repetition of this experiment gave disease reactions for 28, or 56 percent, of the places wounded. Similar studies in which the same number of wounds were made on bean, sugar beet, sedum, and apple, gave, respectively, 34, 100, 100, and 48 percent of infections after incubation periods of about 2 months. This type of inoculation technic was very effective in producing infection, and by means of a scalpel instead of a needle it was largely used in the field experiments on apple trees.

Infection followed the placing of bacteria on the surface of wounds. This was determined by a series of tests. In a preliminary experiment a set of 10 Paris daisy plants were wounded by passing a needle through the stems in each of 5 places. The bacteria were then applied to the wound surfaces in the usual manner with a cotton swab. After 2 months of incubation, 36 of the places wounded, or 72 percent, showed symptoms of the disease. As shown later, the results of the infection-court studies, in which this inoculation technic was used, confirm the results of the Paris daisy experiment. These results demonstrate that infection takes place whether the bacteria are applied to the stem before or after the wounds are made and are in accord with those of Riker (*21*) in experiments with the crown-gall organism.

Because of the importance of wounds in bringing about infection, grafting time is an important period for the infection of piece-root-grafted apple trees. Siegler and Piper (*35*) discuss this point. Whether the bacteria commonly do gain entrance at this time is of such importance that it is discussed in a separate paper, on studies of the seasonal development of the disease, by Riker and Hildebrand (*28*). The reactions at the graft union present so many complications that certain phases of this difficult problem have been simplified in the present work through studies of wounds on the scions of actively growing trees.

Influence of Type of Wound

In a series of trials the type of wound apparently made no difference in the kind of overgrowths but did influence somewhat their rate of development and the percentage of wounds that became infected. A preliminary study on a comparison of needle-puncture and scalpel-cut wounds was made on underground stems of first-year apple trees to test their influence in producing infection. Five needle-puncture wounds were made in each of 10 different trees. An equal

number of scalpel cuts were made in 10 trees. The procedure was to push the scalpel or needle deep into the stem, and to smear the hairy-root bacteria promptly over the wound with a cotton swab. Host injury was obviously greater from the scalpel cuts, and after an incubation period of 2 months infection seemed to be more pronounced from the scalpel injuries. Subsequently however, this difference gradually diminished. In this experiment all the injured trees became infected. The percentages of infection resulting from needle-puncture and scalpel-cut wounds were 38 and 62, respectively. In the percentage of wounds infected there was a discrepancy between the two types of wounds, and this required further consideration.

A more extensive wound-type study was made to determine whether the hairy-root bacteria could enter apple stems through different types of injuries. Three types of wounds were employed, as follows: (1) Scalpel cut, which was made by pushing a scalpel deep into the stem at an angle; (2) bruise, which was made to resemble cultivation injury by striking the stem with a hammer; and (3) needle puncture, which was made by thrusting a needle deep into the center of the stem. The effects of these injuries were studied on first- and second-year trees. Each type of wound was made in 5 places on the stem below ground on 13 trees of both ages. In each case the hairy-root bacteria were promptly applied to the surface of the wounds on 10 trees. The wounds on 3 trees were left untreated, as controls. Ten weeks later the results were taken. Because of the similarity of reactions on the first- and second-year trees, they are summarized together. For the respective types of injury the percentages of wounds becoming infected were as follows: Scalpel cut, 71; bruise, 66; and needle puncture, 41. Of the control wounds 5 percent of both the scalpel and bruise injuries became infected. The infection of the control wounds might be attributed to some soil vector, such as insects, which had free access to the wounded places. From this study it is apparent that all the different types of injuries served as infection courts. Moreover, the symptoms produced by the infection of the different wounds were practically identical except for those in the needle-puncture injuries, which differed from the others in having (1) a slightly longer incubation period, (2) a somewhat lower percentage of infections, and (3) smaller reactions.

A repetition during 1931 of the experiment testing the three types of wounds (scalpel cut, bruise, and needle puncture) on second-year trees gave results similar to those just described. Strips of adhesive tape were placed over all the places wounded as a protection against the possible interference of soil fauna. Scalpel-cut and bruise wounds again were the most effective infection courts for the hairy-root bacteria, as 82 and 66 percent, respectively, of these wounds became infected. Thirty-six percent of the needle-puncture wounds became infected. All the control wounds were negative. The slightly longer incubation periods for smaller wounds suggested the need for further study of the size of wound in relation to the development of the disease.

Extremely shallow wounds were found to be poor infection courts. In 1930 two types of wounds were tested, surface and scalpel cut. The surface injury consisted of a very shallow scraping of the stem to expose the outermost cortex layers to the bacteria. The scalpel cuts were made as before. Each type of injury was made in 5 different

places on each of 40 trees. The wounds on 30 of the trees were inoculated with the bacteria, those on 10 trees being left as controls. All the wounds were covered with adhesive tape. After an incubation period of 10 weeks, 65 percent of the scalpel wounds and only 3 percent of the surface wounds had become infected. An identical experiment repeated in 1931 gave confirmatory results, as 82 and 4 percent, respectively, of the two types of injuries became infected. These results show that shallow injuries to the stems of young apple trees are relatively poor infection courts and that, within limits, the depth as well as the size of wounds is a factor in the amount of hairy-root infection.

Length of Time Wounds Remain Open to Infection

The length of time that wounds remain open to infection was next considered. Preliminary studies were made on Paris daisy, sedum, and bean plants in the greenhouse. The temperature of the greenhouse was approximately 22° C. and the relative humidity about 70 percent. In the first series 5 needle-puncture wounds were made in the stems of 22 Paris daisy plants above, at, and below the ground level. At intervals of 1 hour and 1, 2, 3, 4, 5, 6, 7, 8, 16, and 32 days after wounding, a water suspension of a culture of the hairy-root bacteria was applied to the wounds of two plants with a cotton swab. The results were taken after an incubation period of 2 months. It was found that, in this trial in which the plants were kept on an open greenhouse bench, wounds older than 3 days did not become infected. This indicated that wounds 4 days old had formed a barrier sufficient to keep out the organism. Repetitions of the experiment on Paris daisy, bean, and sedum showed that for 3, 2, and 4 days, respectively, the wounds remained open for infection under greenhouse conditions.

These studies were then extended to include the effect of humidity on the time that infection courts remain open in Paris daisy and bean plants. The same time intervals and number of wounds were employed as in the previous tests. Exposure for 2 days before wounding to a relative humidity of approximately 90 percent at the usual greenhouse temperature of about 22° C. caused the wounds to stay open longer for infection. The period was extended to 5 days for both Paris daisy and bean. In a similar experiment, with the same preliminary treatment, on Paris daisy plants held at the same humidity for 9 days after wounding, the period that wounds remained open for infection was increased to 6 days. These results show that wounds on plants in the greenhouse are ordinarily open to infection for only a few days after they have been made and that raising the relative humidity lengthens this period.

The length of time that wounds on apple remained open for infection was approximately 2 days. Field studies on actively growing apple trees in the nursery were made during the seasons of 1929 and 1930. In 1929 three series of infection-court trials were made, starting, respectively, on May 9, June 7, and July 3. In each series, on the day of starting, 5 wounds were made on the underground stems of each of 4 first-year trees for each interval. The intervals tested were 1 hour and 1, 2, 4, 8, 16, 32, and 64 days. Four wounded trees were kept as controls. At the stated time after wounding, a fresh culture of the hairy-root bacteria was applied to the wounds with a

cotton swab. At the close of the season results showed that the wounds on these trees remained open infection courts for no longer than 2 days. These data corresponded closely with those for greenhouse plants. The exact time periods for May, June, and July were, respectively, 2, 2, and 1 days. In this experiment the time of year appeared to influence the results slightly. An identical experiment was conducted on second-year trees with almost the same results. However, in this case, in the June series, 2, or 10 percent, of the control wounds and 5, or 7 percent, of the wounds inoculated later than 4 days after wounding became infected. These results caused the writer to suspect the interference of insects.

Similar studies in 1930 consisted of five series, starting, respectively, on May 12, June 1, July 1, August 1, and September 1. The procedure of the previous season was followed throughout except that the unnecessary 32- and 64-day intervals were discarded. Both first- and second-year trees were used. To prevent possible interference from hairy-root bacteria or insects that might be present in the soil, adhesive tape was placed over the wounds promptly after they were made. The results (table 3) are in accord with those of the season before on the first-year trees, and fix the time that wounds ordinarily remained open for infection at approximately 4 days for the month of May and 2 days for the other months. Since the application of the adhesive tape over all the wounds the second season eliminated chance infections other than those shown in the table, it appears reasonable to assume that the tape was a barrier to some wound-producing element in the soil environment. The fact that the weather was more favorable for host growth during May 1929 than during May 1930 would seem to account for the discrepancy of 2 days in the length of time the wounds remained open for infection. Figure 1, *B*, illustrates the typical hairy-root infections for the May series, when the plants were 24 weeks old.

TABLE 3.—*Length of time at different periods in the growing season of 1930 that wounds on 1- and 2-year-old apple trees remained open infection courts for the hairy-root organism* [a]

Period between wounding and applying bacteria	Wounds on trees of indicated age infected during—									
	May		June		July		August		September	
	1 year	2 years	1 year	2 years	1 year	2 years	1 year	2 years	1 year	2 years
	Number	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*
1 hour	15	18	17	16	16	15	20	18	12	12
1 day	16	16	8	16	12	13	15	9	8	9
2 days	16	16	4	2	11	10	12	10	4	7
4 days	9	9	0	0	0	1	0	0	0	0
8 days	0	0	0	0	0	0	0	0	0	0
16 days	0	0	0	0	0	0	0	0	0	0

[a] Wounds were made on May 12, June 1, July 1, Aug. 1, and Sept. 1. 20 wounds were made in each trial. Observations were made on Nov. 10.

The studies on the length of time that wounds remain open infection courts both furnish support to and receive support from similar studies on whether callus may be ordinarily an infection court. Callus was found ordinarily to be a barrier against the invasion of the hairy-root organism. Riker and Keitt (*30*) earlier reported that callus formed on apple grafts was not commonly an open infection

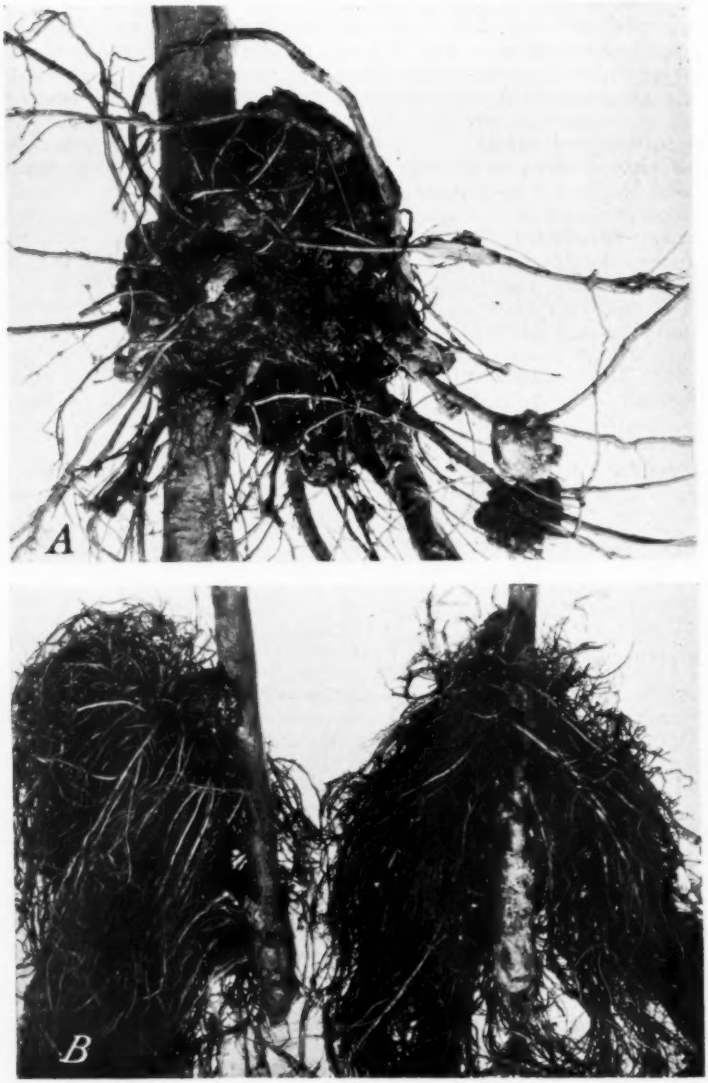

FIGURE 1.—*A*, Hairy-root overgrowths on the lateral roots of a tree infected on the main stem. These lateral overgrowths, which yielded the hairy-root bacteria, presumably followed insect wounds. × ⅗. *B*, Hairy-root infections 24 weeks after inoculation. The bacteria were applied to the specimen on the left 1 hour after the wounds were made and to the specimen on the right 1 day after the wounds were made. × ½.

court for the crown-gall organism. Siegler (*34*) stated that "the graft union after callus has formed is an infection court to a limited extent for the apple organism." Siegler and Piper (*35*) reported a confirmation of Siegler's earlier results, stating that "the grafts are most susceptible at the time they are made and become increasingly resistant with the progression of callus formation."

CALLUS AS A BARRIER TO INFECTION

The infection of callus was studied on apple trees in the nursery. Callus formation was stimulated by wounding the stem, and its reaction toward the application of the bacteria was tested in the soil under natural conditions. Callus of different ages was stimulated by making five wounds below ground on each of eight trees weekly for 10 weeks. On the last date of wounding the bacteria were applied to the wounded surfaces of one half the number of trees. Examination 2 months after inoculation revealed that only those trees wounded on the date of inoculation had become infected. Of these, 11 out of 20, or 55 percent, showed the disease symptoms. The entry of the bacteria in this case had been accomplished before callus had had time to form. Comparable results were obtained in a study by Riker, Hildebrand, and Ivanoff (*29*), similar to that just described except that the callus was stimulated above ground in glass cylinders under approximately aseptic conditions. Similar studies, except for minor changes, were made in 1930. The time, originally 10 weeks, was extended to 12 weeks. Strips of adhesive tape were applied over the wounds to exclude possible soil bacteria and insects. Otherwise the procedure was the same. Again wound callus more than 1 week old did not become infected. Nine out of the 20 wounds inoculated on the day of wounding, or 45 percent, showed symptoms of the disease before callus had had time to form. The results of the season before were verified.

Further studies of callus as an infection court were made in 1931. In one experiment several turns of wire were wrapped around the underground stems of eight trees at weekly intervals for 12 weeks. One week later for each interval, the wrapped portions of the stems of four of the trees were smeared with the bacteria. The other four trees were left untreated, as controls. Observations were made 9 weeks after the application of the bacteria. Although disease symptoms appeared in callus developments of all 12 ages, in 10 percent of the inoculated trees and in 8 percent of the control trees, this study seemed to demonstrate that callus is not commonly an infection court. The fact that a white grub was observed feeding on one of the overgrowths which later showed infection indicates that the chance infections encountered may have been due to insect injuries.

In another experiment callus was stimulated by making a slanting upward cut with a scalpel into the underground stems. To prevent reunion of the several tissues a strip of adhesive tape was inserted in each cut. In all other particulars this experiment was identical with the preceding one. When the observations were made, it was found that 12 percent of the inoculated trees were infected and that the control trees were not infected. These results appear to substantiate those just reported in showing that unwounded callus does not ordinarily serve as an infection court.[4]

[4] Illustrations of these callus developments will be found in a paper by Riker and Hildebrand (*28*).

Since callus is very easily injured, a study was made of the susceptibility to hairy-root infection of callus that had received small wounds. Shallow wounding did not commonly, if at all, permit the entry of the hairy-root organism. Using the technic previously described, the writer stimulated callus by wounding under three conditions: (1) On underground stems exposed to the soil; (2) on underground stems protected by adhesive tape; and (3) on aerial stems in glass cylinders (*29*) protected from outside contamination. At 4 biweekly intervals 5 wounds were made in each of 2 plants for each of the 3 conditions named above. One week after the last wound was made the callus developments of the various ages were lightly pricked and scratched so as to limit the injury to the outer few layers of callus cells. The bacteria were then smeared over the injured surfaces. After an incubation period of 8 weeks only one of the wounds, and that in a 1-week-old callus, was observed to be infected. In this case it appeared that, after the injury was made, insufficient callus remained over the susceptible host tissues to be a barrier to the organism. Parallel to this an equal number of wound inoculations were made which resulted in the development of the disease. Repetition of one part of this experiment, namely, that on the underground stems protected by adhesive tape, gave the same results the next season. These results indicate that shallow wounds in callus do not ordinarily bring about infection, which is in accord with the results obtained when shallow wounds were made in apple stems.

INFLUENCE OF SOIL INSECTS

Wounds produced by insects should be considered as a possible factor in infection by the hairy-root organism in the nursery. Root-chewing arthropods have been found by Banfield (*4*) associated with the occurrence of crown gall on raspberry. He showed that healthy raspberry plants grown in soil which had been inoculated with the crown-gall organism but which was free from insects became diseased only when white grubs were introduced into the controlled environment. Consequently it seemed desirable to examine the possible relation of insects to hairy-root infection.

Root-feeding insects have been observed during three growing seasons in the soil about nursery apple trees. Preliminary to a more detailed study (*28*) of seasonal development of diseases, including hairy root, a survey was made of the insects commonly present around the graft unions in the soil. From the survey it was determined that root-feeding insects, especially white grubs (Phyllophaga) and wireworms (Elateridae), were commonly present in the nursery soil during the growing season, from May to October.

Insects eating both hairy-root and healthy tissue have frequently been seen during the observations of diseased and healthy trees. These observations were made in the course of studies which required the examination of several hundred young apple trees each week. On several occasions during the growing season, from May to October, white grubs were observed feeding upon hairy-root overgrowth tissue and other underground parts, such as stem tissue, root tissue, and both infectious and noninfectious hairy roots. Observations in 1931 served to support the findings of the two previous seasons as to the activity of this insect and pointed to it as an important agent in

producing wounds that might lead to the hairy-root disease if the causal bacteria were present.

Injuries similar to those produced by white grubs in Kansas have been observed in Wisconsin, Iowa, Missouri, Nebraska, and Oklahoma. Wireworms also were occasionally observed burrowing into callus and overgrowth tissues, especially in the earlier stages. Small fungus-gnat larvae (Mycetophilidae) were found to frequent some of the enlargements in the crevices and sometimes the tissues. On three occasions white ants were found making trenches in the main root or in the large branch roots, sometimes for the greater portion of their length. Twice microscopic examination of the surface of five young overgrowths revealed the presence of nematodes. These are but some of the instances of interference by soil fauna with the underground plant parts as observed in the nursery during the growing season.

Over a period of 4 years from one to several specimens of infected lateral roots were observed in positions that could have been reached only by insects. Riker and Hildebrand (*28*) in their studies found from 0 to 0.6 percent of the lateral roots infected. Hairy-root bacteria were isolated from the small overgrowths and from the main overgrowth in the specimen illustrated in figure 1, *A*, proving their infectious nature.

The development of new infections during both the first and the second growing season in Kansas was most easily explained as due to the agency of insects. As stated earlier, wounds appear to be necessary for the entrance of the bacteria. To account for the occurrence of new infections over so long a period, some wound-producing agency must, therefore, have been almost constantly present in the soil around the graft unions. Two examples may be cited. In a block of 240 trees, 39 percent were found infected at the end of the first growing season and 59 percent at the close of the second growing season. A summary of the studies over a period of 4 years, in which about 7,000 trees were examined, showed at the close of the second season an increase in disease development of 13 percent over the first season. Further evidence on this point will be found in the paper on seasonal development by Riker and Hildebrand (*28*).

Several experiments were suggested by the observations on insects in relation to hairy root. Isolations of the hairy-root bacteria were made from white grubs that had been feeding on diseased tissue. Out of 38 isolation trials from the alimentary tracts of white grubs in 1930, only one culture of the bacteria was obtained. The method of isolation consisted in disinfecting the exterior of the insect by immersion for 10 seconds in mercuric chloride, 1 : 1,000, and removing the digestive tract, which was transferred directly to 100 cc of sterile distilled water. The vessel containing the water and digestive tract, after being shaken, was allowed to stand for 30 minutes, when five 1-cc portions were plated on bile agar (*19*). Out of five isolation trials in 1931 from the mouth parts of white grubs which had been eating young hairy-root overgrowth tissue when collected the day before and on which no surface disinfectant was employed, two cultures of the hairy-root bacteria were obtained. Eleven isolation trials from the alimentary tracts of as many white grubs, when no disinfectant was employed, were all negative. These studies are of a limited nature. However, the fact that some insects which habit-

ually feed upon underground plant parts may carry the hairy-root bacteria even for a short time is of significance in the life history of the hairy-root organism.

Isolations from other insects gave negative results. Regardless of the significance that may be attached to these isolation studies, the fact remains that wounds produced are potential infection courts for the bacteria.

Insect repellents—paradichlorobenzene (*9*) and mercuric chloride (*7*, *10*)—were employed in an effort to reduce the amount of insect injury and consequently of hairy-root infection on first-year trees in the nursery. Although 50 and 35 percent reduction in hairy root was secured, the data are omitted because they are insufficient for definite conclusions.

Insect barriers made of treated cloth were tested. Cloth of unbleached muslin, 50 meshes to the inch, was washed to remove the filler, and dried. Strips 4 inches wide were folded and sewed to make a sack approximately 1 inch in diameter and 4 inches long. The sacks were soaked for 30 minutes in a preservative solution of 29 parts (by weight) of copper oleate dissolved in 71 parts of gasoline. This treatment has been successfully used in the preservation of cotton fish nets in both sea and lake water (*38*). The sacks were slipped over the graft unions and fastened at the base by folding and securely wrapping with a strip of adhesive tape 4 inches long by one half of an inch wide. Autoclaved soil was introduced from the top in sufficient amount to keep the cloth from contact with the union. The top of the sack was then folded and wrapped in the same manner as the bottom. Grafts wrapped with string and made from the same supply of scions and roots were used in this experiment. Five hundred grafts were fitted with sacks. An equal number without sacks were planted alongside. The results taken at the close of the season showed about 6 percent of the sacked grafts and 24 percent of the controls infected. The cloth resisted decay until August, when an occasional sack had begun to show disintegration. Along with this treatment there was a growth-retarding action from the use of the sacks of about 4 inches to the tree. Moreover, about 16 percent more of the sacked trees than of the controls died during the summer. Although the reduced amount of disease for the sacked grafts may have been partly due to one or more causes besides the cloth barrier, the evidence appeared to warrant a repetition of this experiment. The following season a similar study gave more reliable results. For the sacked and control trees there were, respectively, 13.9 and 26.0 percent of disease, 75 and 76 percent of stand, and 32.6 and 34.4 inches of average height. With so little difference in stand and height, the difference in percentage of disease appears important. The possibility of antiseptic action by copper oleate upon the hairy-root bacteria was tested but was not found to be significant. These experiments gave added strength to the idea of insect interference.

INFLUENCE OF CONDITION OF HOST

The entrance of the hairy-root bacteria into nursery apple trees was studied in relation to the susceptibility of the host as influenced by (1) the time of the growing season, (2) the age of the tree, (3) the size of the tree, (4) the variety of the tree, and (5) the previous infection of the tree.

The period of the growing season seemed immaterial so far as entrance of the organism was concerned. This was determined by making inoculations at weekly intervals throughout three growing seasons. Each week 10 trees were inoculated in the usual way by introducing the bacteria through scalpel cuts in the stems in five places. The percentage of trees that became infected was used as the measure of the entry of the bacteria. The results for the three seasons are given in table 4. Except at sporadic intervals during the growing seasons studied, the trees permitting entry of the bacteria approached 100 percent, the averages for 1929, 1930, and 1931 being 97, 96, and 98 percent. The apparent exceptions seem to be within the range of experimental error. The rapid falling off in the percentage of disease in September 1929 was probably due to the early dormancy of the trees. Of the inoculations made on September 4 and September 11, 50 and 0 percent, respectively, had become infected by the time of the last observation in November. This idea was verified when in May 1930 it was found that all the trees which had apparently become dormant the September before showed infection. Consequently, 100 percent infection is shown in table 4. The bacteria had entered and overwintered in the wounds, ready to produce infection the following spring. Corresponding inoculations in September 1930 and September 1931 produced disease symptoms in November. It appears, therefore, that bacteria will enter the host during any part of the growing season and will produce infection if the trees have not become dormant.

TABLE 4.—*Wounded apple trees infected by hairy-root bacteria at different periods during 1929, 1930, and 1931*

Date	Trees infected [a]	Date	Trees infected [a]	Date	Trees infected [a]
1929	*Percent*	1930	*Percent*	1931	*Percent*
May 15	90	May 12	100	May 11	100
May 22	100	May 19	100	May 18	100
May 29	100	May 26	100	May 25	100
June 5	100	June 2	100	June 1	100
June 12	100	June 9	100	June 8	90
June 19	90	June 16	50	June 15	100
June 26	100	June 23	100	June 22	100
July 3	100	June 30	100	June 29	90
July 10	100	July 7	100	July 6	100
July 17	90	July 14	100	July 13	100
July 24	100	July 21	100	July 20	100
July 31	100	July 28	100	July 27	100
Aug. 7	100	Aug. 4	90	Aug. 3	100
Aug. 14		Aug. 11	90	Aug. 10	100
Aug. 21	100	Aug. 18	100	Aug. 17	90
Aug. 28	80	Aug. 25	100	Aug. 24	90
Sept. 4	[b] 100	Sept. 1	100	Aug. 31	100
Sept. 11	[b] 100	Sept. 8	100	Sept. 7	100
				Sept. 14	100

[a] 10 trees were inoculated each week.
[b] For explanation see paragraph above table.

The age of the nursery apple tree, in this region where trees are grown only 2 years, did not seem to influence susceptibility to infection by the hairy-root bacteria. Inoculations made in 1929 on first- and second-year trees showed 85 and 93 percent of infection, respectively, and inoculations made in 1930 and 1931 showed 97 percent of infection on both first- and second-year trees. An average of 40 trees, each wounded in five places, was used in each part of the test.

All sizes of growing apple trees were found infected in the same field under natural conditions. Each year, at the end of the growing season, representative first-year trees were selected, examined for disease development, and measured for height. A graphic presentation (fig. 2) of the data accumulated during the 1929 season serves to

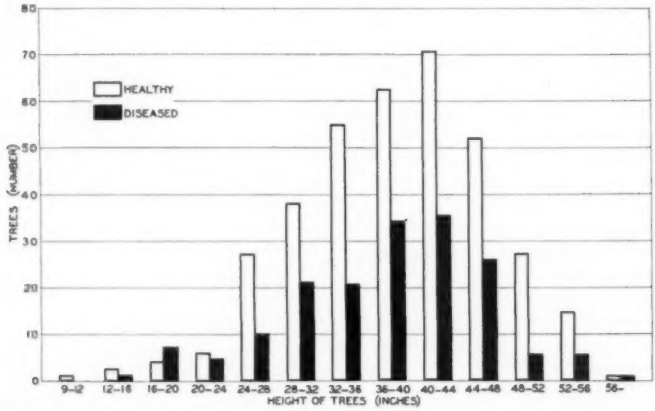

FIGURE 2.—Distribution in 1929 of healthy and hairy-root diseased 1-year-old apple trees in groups based on height of trees in inches.

bring out the distribution of the diseased and healthy trees with respect to the different size groups arbitrarily chosen. For graphing, all the trees were placed in size groups differing from each other by intervals of 4 inches, e.g., (1) less than 12 inches, (2) 12 to 16 inches, (3) 16 to 20 inches, etc. These data showed a fairly even distribution of both diseased and healthy trees over the range of size groups. A similar study on second-year trees (fig. 3) gave similar results. Since all

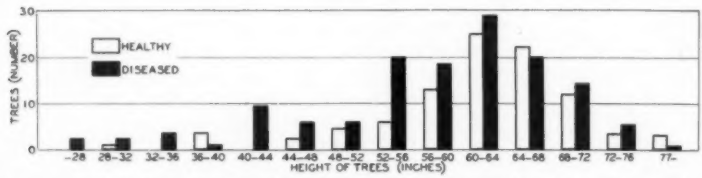

FIGURE 3.—Distribution in 1930 of healthy and hairy-root diseased 2-year-old apple trees in groups based on height of trees in inches.

sizes of trees may be found infected in the field it seems logical to conclude that size is not an important factor in susceptibility of trees to infection by the hairy-root organism. Further evidence in support of this conclusion was derived from an experiment in which 311 trees as they came in the row were inoculated; subsequently, 95 percent were found to have become infected. When compared as to size it was found that most of the 5 percent which resisted infection fell into the more numerous middle-sized groups.

Varietal susceptibility to infection by the hairy-root organism differed widely among the varieties of trees studied. That not all

varieties of apple are equally susceptible to the hairy-root disease has been recognized by different workers, including Hedgcock (*12*), who reported as the result of a country-wide survey that the Ben Davis, Wolf River, and Northern Spy varieties especially were attacked in the nurseries. The present study was made on the relative susceptibility of the varieties of apple trees commonly grown at Topeka, Kans. Late in May 50 inoculations were made in each of 29 varieties in 1930 and in each of 37 varieties in 1931. Twenty-seven of the same varieties appeared in the tests both years. The varietal names were checked with "Standardized Plant Names" (*2*), the local names being retained when the variety did not appear in the standard list. Two names are given for some of the varieties. After 2 months of incubation the overgrowths were cut off even with the stem surface, wrapped in moist paper, and taken to the laboratory. The following points were recorded for each variety: (1) The number of inoculations giving positive reactions, (2) the volume displacement of hairy-root tissue, (3) the wet weight of hairy-root tissue, and (4) the dry weight of hairy-root tissue.

On the basis of the percentage of wounds infected, the different varieties ranged in susceptibility from 12 to 100 percent in 1930 and from 22 to 100 percent in 1931 (table 5). Three of the 27 varieties appearing both seasons (Fameuse, Florence (crab), and Livland Raspberry) gave the same percentage of positive inoculations; three others (Hopa (crab), McIntosh, and Wealthy) gave a lower percentage of positive inoculations the second season; the remaining 20 varieties showed an increase in the percentage of positive inoculations the second season. For example, the Yellow Transparent variety had a susceptibility to infection of 48 percent in 1930, and 82 percent in 1931. On the basis of growth, the varieties showed some difference with respect to one another and considerable difference with respect to the season. The average amount of hairy-root growth for each positive inoculation was calculated on the three bases already given.

Since the three different measures of hairy-root development showed such a close correlation, only the first one need be discussed. The volume growth in cubic centimeters of the different varieties ranged from 0.31 to 1.71 in 1930 and from 1.00 to 4.04 in 1931. Without exception, in the 27 varieties appearing both seasons a considerably larger growth of diseased tissue occurred the second season. When the varieties were grouped the average growth of diseased tissue in 1931 was approximately 300 percent of that of 1930. These studies indicate that varietal differences in susceptibility to the hairy-root organism are important factors in the infection and development of hairy root.

In 1929 50 second-year trees which had become diseased the first season were wounded by deep punctures made with a pointed blade above, below, and at either side of the union, at least 1 cm from the enlargements; 25 other diseased trees were kept unwounded as controls. A group of 25 healthy trees were wounded about the union in the manner described above; 50 others were left unwounded as controls. The soil in which the trees were growing was infested with the hairy-root bacteria. Of the diseased trees, 24 percent of those wounded and 12 percent of those left unwounded showed new infections at the end of the year; of the healthy trees, 28 percent of the wounded and 14 percent of the unwounded became infected. This

study revealed the fact that new infections occurred whether the trees were diseased or healthy. Moreover a number of new infections occurred in the same series where no wounds were made, the majority of these infections appearing at the union or above it on the scion. Numerous observations in the field confirm these findings. Apparently previous infection of nursery apple trees does not produce an immunity to later infection. This is in accord with the results obtained by Smith et al. (*36*), Brown (*5*), and Riker (*22*), in their work with crown gall.

TABLE 5.—*Results of tests made during 1930 and 1931 in Kansas on the relative susceptibility of 38 varieties of nursery apple trees to infection by the hairy-root organism* [a]

Variety	Inoculations positive		Average growth of reactions measured by—					
			Displacement		Wet weight		Dry weight	
	1930	1931	1930	1931	1930	1931	1930	1931
	Per-cent	*Per-cent*	*Cc*	*Cc*	*Grams*	*Grams*	*Grams*	*Grams*
Anoka	34	76	0.60	1.58	0.51	1.32	0.12	0.27
Arkansas [b]	20	56	.65	1.21	.50	1.06	.10	.18
Baldwin	42	86	.31	1.16	.26	1.05	.05	.21
Beauty (crab)		34		1.30		1.03		.19
Ben Davis	72	88	.39	1.89	.38	1.50	.07	.27
Cortland	100		.88		.80		.19	
Delicious	62	94	.45	1.70	.36	1.57	.07	.37
Dolgo (crab)		98		3.51		3.08		.66
Early Cooper	98	100	1.71	3.78	1.40	3.43	.26	.61
Early Harvest	26	68	.69	1.56	.59	1.33	.15	.27
Fameuse [c]	94	94	1.49	3.77	1.41	3.46	.22	.60
Florence (crab)	60	60	1.13	3.33	1.02	3.05	.18	.51
Gano I [d]	30	100	.50	3.04	.45	2.77	.07	.62
Gano II		92		1.72		1.63		.29
Grimes Golden	38	84	.80	1.38	.67	1.24	.13	.27
Hopa (crab)	38	22	.42	1.45	.34	1.07	.08	.20
Hyslop (crab)	98	100	1.39	3.86	1.28	3.72	.25	.76
Jonathan	56	92	.61	2.59	.55	2.39	.10	.47
Livland Raspberry [e]	100	100	1.31	2.14	1.19	1.87	.21	.40
McIntosh	38	36	.60	2.12	.57	2.08	.14	.41
Minkler		70		1.08		1.00		.19
Maiden Blush	86	96	.89	3.17	.78	2.77	.15	.52
Northwestern Greening		90		2.69		2.35		.37
Oldenburg [f]		96		2.73		2.46		.41
Rambo	68		.36		.30		.08	
Red Astrachan	88	98	.60	1.53	.56	1.23	.08	.22
Red June		98		2.96		2.58		.61
Rome Beauty	20	30	.44	1.47	.40	.95	.07	.23
Stayman Winesap	12	86	.33	2.63	.31	2.44	.05	.44
Tolman Sweet	54	84	.46	1.88	.39	1.71	.09	.34
Turley		74		1.65		1.28		.58
Wealthy	74	72	.51	2.22	.49	1.96	.10	.39
Whitney (crab)	14	68	.57	2.44	.46	2.07	.09	.48
Willowtwig		98		2.61		2.31		.39
Wilson June		96		4.04		3.37		.67
Winesap	24	90	.42	2.24	.37	2.03	.07	.44
Winter Banana	60	86	.47	1.60	.40	.87	.08	.15
Yellow Transparent	48	82	.62	2.02	.52	1.83	.11	.37
York Imperial	16	66	.44	1.18	.39	.94	.10	.21
Average [g]	52	78	.69	2.16	.61	1.91	.12	.37

[a] 50 inoculations were made on each variety.
[b] Mammoth Black Twig.
[c] Snow.
[d] Black Ben.
[e] Livland Raspberry.
[f] Duchess.
[g] These averages represent the 27 varieties occurring both seasons.

LOCATION OF ORGANISM WITHIN HOST

The location of the hairy-root organism in the host tissues was another phase of the life history studied. Studies on the location of the crown-gall organism in the tissues of several hosts were made by Riker (*21*), Robinson and Walkden (*32*), and Ivanoff and Riker (*13*). Because of its similarity to the hairy-root organism, the studies on the crown-gall organism suggested methods of approach for this work.

The hosts selected for this study were Paris daisy and apple. Inoculations were made only on young stems of vigorous plants, from 1 to 4 inches below the apex. The method of inoculation was by needle puncture through a drop of a suspension of the bacteria placed on the surface of the stem. Control punctures were made without the bacteria. Inoculated and uninoculated stem specimens were prepared for study, at intervals of 1 hour, 1, 2, 3, 4, 8, and 16 days after treatment. This material was examined both in the fresh state and when embedded in paraffin after being fixed in formalin-acetic-alcohol. Parallel series were prepared in which the inoculum consisted of hairy-root bacteria mixed with *Phytomonas insidiosa* (McCulloch) Bergey et al., a Gram-positive organism, and hairy-root bacteria mixed with india ink. These mixtures, devised to aid in the location of the bacteria in the tissues, are similar to those used by Ivanoff and Riker (*13*). Of a large number of staining combinations tried, including the use of Gram's stain in studying the mixture of organisms, the staining procedure found most satisfactory was to immerse the preparation for about 1 second in dilute safranine, 1:1,000, followed by the light-green counterstain.

Relations of the hairy-root organism with the host are apparently first begun in the liquid released by the wounds. When wounds were made in the stems of Paris daisy and apple, there was a water-soaking of the neighboring tissues similar to that described by Riker (*21*) in tomato. These juices, liberated from the cells by the wound, flooded the neighboring intercellular spaces for a short distance from the wound cavity. The bacteria were observed in the wound cavity and for some distance between the cells in the surrounding host tissues. The bacteria, operating under a complexity of forces, as suggested by Ivanoff and Riker (*13*), become distributed in the wound juices that ordinarily fill the neighboring intercellular spaces.

In the fresh material the bacteria appeared to be located in the intercellular spaces in both Paris daisy and apple stems. For example, 8 days after inoculation a comparison of both Paris daisy and apple tissues which had received control punctures was made. The crushed tissues bordering the wound cavity in both inoculated and uninoculated stems were discolored. The inoculated stems had an intercellular discoloration spreading out into the tissues for a short distance from the path of the needle. It varied from a pale yellow to a brown and was especially marked in the tissues outside the cambium. None of the uninoculated tissues showed this discoloration. From the limited studies made the position of the discoloration, whether in the walls or in the material between the cells or in both, could not be determined with finality. However, in the section examined, it seemed to be present in both places in varying amounts. The extent of the discoloration between the cells from the edge of the wound seemed to correspond rather closely to the area which appeared water-soaked when the

wound was made, roughly a distance of 10 to 20 cells. The fact that outside the path of the needle only the inoculated tissue became discolored indicated that this condition was caused by the presence of the bacteria.

The fixed Paris daisy material, whether stained or unstained, was found to contain the bacteria in the spaces between the cells. Sections were cut from 10μ to 20μ in thickness. After inoculation the unstained tissues showed discolorations similar to those of the fresh material. A yellowing of the regions apparently invaded by the bacteria became evident 4 days after inoculation; 8 days after inoculation the color became deeper, almost brown. Staining dyes showed a strong affinity for these regions, so that the bacteria, if present, were obscured. In some sections the use of india ink or Gram-positive bacteria made the invaded intercellular spaces more distinct, although their position and extent seemed to be the same. It was generally found that the discolored regions in 8-day-old preparations took the dyes very readily, making the bacteria difficult to see. Starting from the wound, search was made for the bacteria. Only cells that had been injured were observed to contain bacteria within them (fig. 4, *A*). The bacteria could be traced for short distances into the intercellular spaces. In the pith of Paris daisy there are comparatively large intercellular spaces. Photomicrographs of a cross section and longitudinal section of stems 8 days after inoculation showed deeply stained material in these intercellular spaces (fig. 4, *B* and *C*). With high magnification the bacteria are visible in such locations (fig. 4, *D*).

Studies of the apple stems involved preparations similar to those on Paris daisy, and showed the bacteria to be similarly situated. The extent of migration of the bacteria into the tissues outside the xylem from the path of the wound seemed even more limited than for Paris daisy. Moreover the bacteria were observed to stimulate hyperplasia only in the tissues outside the xylem.

The presence of the organisms between the cells of the tissues bordering the wound stimulated a multiplication of the cells in those localities. This change appeared to begin about 8 days after inoculation. All the tissues were examined less than 16 days after inoculation. Cross sections of stems made 8 to 16 days after inoculation revealed the presence of circular areas of hyperplasia (fig. 4, *E* and *F*) outside the xylem. Variable in number and from one to several cells in radius, these areas were in close proximity to the wound borders and appeared to be about the intercellular spaces containing the bacteria. For a distance of from 1- to 10-cell diameters away from the center of such localities the cells were stimulated to division in a tangential plane. This was the condition 16 days after inoculation. Bacteria have been observed usually at the centers of such areas, which when stained stand out distinctly in strong contrast to the surrounding tissues. The development of hairy root after the formation of the hyperplastic areas is an important phase of the disease requiring further investigation.

In the injured xylem vessels the organisms appeared for a short distance from the wounds in both cross and longitudinal sections of apple and Paris daisy stems. In some longitudinal stem sections of Paris daisy it was common for the bacteria to be present in the vessels to the end of the section. Stem cross sections above and below the wounded area showed the bacteria to be present within the vessels

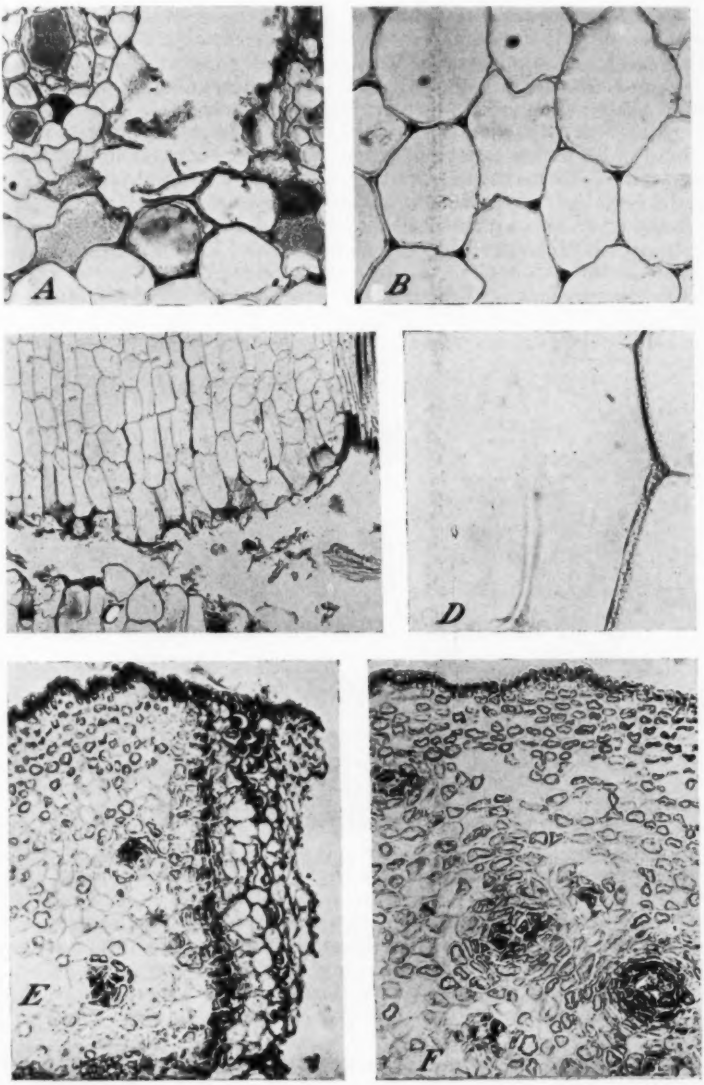

FIGURE 4.—Photomicrographs showing the location of the hairy-root bacteria: *A*, Cross section of a Paris daisy stem 4 days after puncture inoculation, showing the bacteria within injured host cells and in the neighboring vessels. × 270. *B*, Cross section of a Paris daisy stem 8 days after puncture inoculation, showing discolored intercellular spaces which contain the hairy-root bacteria. × 270. *C*, Longitudinal section of a Paris daisy stem 8 days after puncture inoculation, showing discolored intercellular spaces which mark the position of the hairy-root bacteria. × 100. *D*, Longitudinal section of a Paris daisy stem 8 days after puncture inoculation, showing the hairy-root bacteria in the intercellular space. × 550. *E*, Cross section of an apple stem 8 days after puncture inoculation with the hairy-root organism, showing the position of the circular areas of hyperplasia in relation to the host tissues and to the wound. × 200. *F*, Another cross section of an apple stem showing the position of the circular areas of hyperplasia in the host tissues above and below the wounds. × 200.

(fig. 4, *A*). Because no changes in these tissues were observed, the presence of the bacteria in the vessels appeared to be of no importance in bringing about infection.

Several thousand inoculations made on unwounded apple trees failed to produce infection. Symptoms of hairy-root infections induced by inoculation have invariably originated at the point of inoculation. This characteristic of hairy root is in accord with the limited migration of the bacteria from the wound cavity found in the studies on the location of the bacteria.

A study of the location of the bacteria in the older tissue was continued with bacteriological technic.

The bacteria seemed to be most abundant in the soft outer tissues of the enlargement. This was determined from isolation studies in which the writer used the technic described earlier. The interior of the basal enlargements of hairy root consisted chiefly of xylem ele-

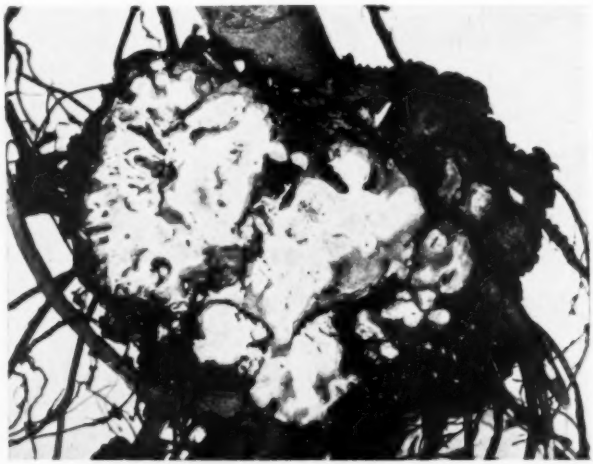

FIGURE 5.—Section of a hairy-root overgrowth after a 15-minute exposure to the air, showing the white, hard interior tissues and the soft outer tissues which have discolored in the air and which usually contain the hairy-root bacteria. Actual size.

ments. For some distance beneath the surface two tissues are paramount in the enlargement—the hard, white, woody interior, and a soft, almost colorless, cortical exterior of varying thickness. Exposure to the air for a few minutes caused these soft outer tissues to become brown as if oxidized. The vascular tissues remained unchanged (fig. 5).

The bacteria were isolated consistently from the surface and subsurface parenchymatous tissues of the enlargements. In order to clarify the position of the bacteria in the enlargements, isolations were made from different tissues. Different kinds of enlargements common to the nursery, approximately 50 percent of which were hairy root and the remainder principally wound overgrowth, were taken at random and isolations made. In a series of 152 isolation trials, 131 series of platings were made from the surface, 152 from the subsurface, and 152 from the deep interior. The surface tissue yielded the largest

percentage of the organism, or 39 percent (table 6). In sampling the surface tissue soil particles were sometimes unavoidably included. The subsurface parenchymatous tissue was removed aseptically and yielded infectious bacteria in 38 percent of the isolation trials. From the deep vascular tissue the bacteria were obtained only from discolored areas where soil particles were imprisoned. It should be noted in the table that nonpathogenic bacteria, *Bacillus radiobacter* Beij. and Van Deld., were uniformly obtained in considerable quantity. The significance of these nonpathogenic bacteria has not been determined. However, the location of the bacteria at the surface of the enlargements is of importance in relation to the soil.

TABLE 6.—*Summary of isolation studies from the different tissues of representative enlargements found in the nursery* [a]

Tissue examined	Isolation trials	Enlargements yielding—		
		Phytomonas rhizogenes	Nonpathogenic bacteria	No bacteria
	Number	*Percent*	*Percent*	*Percent*
Surface parenchymatous	131	39	32	29
Subsurface parenchymatous	152	38	28	34
Deep interior vascular	152	[b] 1	[c] 2	97

[a] No crown galls were included among these specimens.
[b] The deep interior tissue yielded *Phytomonas rhizogenes* only from discolored tissue containing soil particles.
[c] Nonpathogenic bacteria also were obtained only from discolored tissue containing soil particles.

SOIL RELATIONS OF ORGANISM

The bacteria were found to be abundant on the surface of the hairy-root enlargements. The most promising method of studying this problem seemed to be dependent upon the production of infections under approximately aseptic conditions away from common soil contaminants. The production of hairy root under these conditions has been worked out by Riker, Hildebrand, and Ivanoff (*29*), where the technic is given. Examples of the diseased specimens produced by this method are described. Briefly, the technic consisted of fitting glass cylinders around the stems of apple and sealing them so as to exclude all micro-organisms, except the hairy-root bacteria, used in making the inoculations. At intervals of 9, 10, 13, 15, 17, 19, and 21 weeks after inoculation the specimens were taken for study. The surfaces were sterilized by immersion for 15 minutes in a 20-percent solution of a sodium hypochlorite preparation, known as "Bacillikill", which contains 3.5 percent of sodium hypochlorite by weight. The disinfectant was removed by washing through three changes of sterile distilled water. In an effort to recover the organism, bile-agar platings were made of the sterile distilled water in which the specimens were allowed to be immersed for intervals of 10 minutes, 1 hour, 4 hours, 1 day, and 2 days.[5] Hairy-root bacteria were obtained in the washings of all the specimens and for all the washing intervals tested. One control specimen gave off no bacteria; a crown-gall specimen yielded the crown-gall bacteria.

[5] This technic was developed after consultation with W. M. Banfield, Department of Botany, University of Chicago.

A parallel exit study, which was conducted on disease specimens produced in the soil, gave similar results. Four specimens, inoculated, respectively, 2, 4, 6, and 8 weeks previously, were used in this study. These specimens were washed free from soil particles in such a manner as to produce no injuries. They were then sterilized and washed free from the sterilizing solution as in the preceding experiment. The specimens were placed for 1 hour in sterile distilled water before the water was plated. After an incubation period of 1 week the hairy-root bacteria were obtained from the platings of specimens of all four ages. The proof of the identity of the bacteria saved from these isolations was established by positive inoculations on apple.

The bacteria were found rather often in the soil near the hairy-root formations. In 1929, 10 isolation trials were made at each of three 10-day intervals, starting May 28. The technic employed was as follows: For each trial a sample of about 500 g of soil was removed from around a hairy-root formation and taken to the laboratory. After a thorough mixing, a sample of 20 g was weighed into a flask containing 200 g of sterile distilled water. This was shaken and allowed to settle for about 1 hour. One cubic centimeter of the supernatant liquid was then transferred to each of three Petri dishes. Loop dilutions from each of these were made to three other dishes, respectively, containing 1 cc of sterile distilled water. Bile agar was added, and the plates were then allowed to incubate at room temperature for 1 week, after which an examination was made. The percentage yields of the hairy-root organism in the three successive trials of this experiment were 40, 80, and 60, respectively. The bacteria were obtained in 18 of the 30 attempts, or 60 percent.

A similar study, consisting of 10 trials conducted on soil taken from around healthy unions, were all negative. Since the bacteria were obtained relatively easily from random samples from the nursery soil this may seem unusual. However, in securing these samples soil was taken from around trees of a poorly knotting variety where there was less chance of soil contamination.

A 3-day-old growth of bacteria from six large prescription bottles was poured into a cubic foot of soil stored in the field, and the same treatment was given a similar amount of soil kept in a storage cellar. These soils were inoculated in October 1930, and three isolations were subsequently made from each soil at monthly intervals, through April 1931. The soil stored in the cellar yielded the bacteria in 20 out of 21 trials; the field soil yielded the bacteria in 19 out of 21 trials. It is apparent from these experiments that the bacteria may overwinter in soil kept in the field or with nursery stock stored in the cellar.

The longevity of the bacteria in field soil has been found to exceed 1 year both in Wisconsin and in Kansas. At Madison, Wis., steamed soil inoculated with the hairy-root bacteria in the summer of 1928 yielded the bacteria in 2 out of 4 trials in April 1929 and in 1 out of 4 trials in October 1929. Field soil inoculated in the summer of 1929 yielded the bacteria in 1 out of 3 trials in October 1930, and in 0 out of 3 trials in April 1931. In 1930 an attempt was made in Kansas to recover the hairy-root organism from the soil of four fields that had grown or were growing nursery apple trees. Fields 1, 2, 3, and 4 had grown trees during 1927 and 1928, 1928 and 1929, 1929 and 1930, 1930 and 1931, respectively. At five different intervals (June 3, June 17, July 11, July 30, and August 28), 10 soil samples from each

field were collected at random from a depth of about 4 inches and then mixed for each field. Platings were made according to the technic of the previous season, except that four instead of three 1-cc samples were taken for each flask. The results show that the bacteria were present but were not very abundant in three of the fields. Briefly, from a total of 20 trials for each field, the hairy-root bacteria were obtained in 0, 20, 25, and 30 percent, respectively. Only in field 1, from which the trees had been removed 2 years previous, were no bacteria obtained. The presence of the bacteria in field soil from which the trees had been removed a year previous (field 2) and their absence in soil which had grown trees 2 years previous (field 1) indicated the length of time after inoculation that bacteria could be isolated from the soil. Isolations were again attempted in May 1931 from fields 2, 3, and 4 and from the soil of the new graft field, which the season before had grown apple seedlings. Field 2 failed to yield the bacteria, although it had done so the previous season. Fields 3 and 4 and the new graft field yielded the bacteria in 25, 25, and 50 percent of the trials, respectively. The presence of the bacteria in the graft field in May in so short a time after the grafts were planted may perhaps be traced to the growth of seedlings in this field the previous season. These results correspond in general to those secured by Patel (*20*) and Banfield (*3*) for the length of time crown-gall bacteria could survive in unsterilized soil.

DISTRIBUTION AND TRANSMISSION OF ORGANISM

Although nursery stock may be inspected and diseased trees destroyed, it is difficult to find the incipient stages and impossible to find recent infections. Moreover, healthy plants may carry the bacteria on their roots. In 1929 a consignment of apparently healthy Wealthy apple grafts were planted at Madison, Wis. (*23*), in beds of soil that had been steamed. At the close of the growing season some of the trees had overgrowths at their unions. Twenty trees showing these malformations were selected at random. Isolations from 14 yielded typical hairy-root bacteria. The probability that these bacteria entered the trees from the steamed soil is remote, hence they were probably carried either in the unions or on the surface of the roots. It seems fairly obvious that the part played by shipments of nursery stock is important in the widespread dissemination of the disease. This may account for its presence in practically every region where the host plant is grown.

The seedling root was found to be one medium of transmission of the causal organism from one crop to the next. Siegler and Piper (*35*) reported that "the seedlings may in nature carry surface-borne organisms in quantities sufficient to be considered an important source of inoculum." Isolation trials from washings from certain seedling roots have yielded the bacteria. Three-inch pieces from 26 different seedling roots were placed in test tubes partly filled with sterile distilled water. Platings were made from the water 4 hours later. Hairy-root bacteria were obtained from 24, or 92 percent, of the specimens, indicating that the seedling is at least one of the primary sources of inoculum. This and other evidence obtained from these life-history studies points to grafting time as the time when the principal primary infection of the apple tree takes place. The presence in the soil of

primary infections, resulting from the entrance of the bacteria at grafting time, together with the various wound-producing agencies, are the factors which would seem to account for the secondary spread of the disease.

SUMMARY

In studies of infectious hairy root special consideration has been given to the life history of the causal organism in relation to its pathogenesis on nursery apple trees.

The differentiation of hairy root from crown gall and from wound overgrowth has been repeated and confirmed.

The entrance of the bacteria into the host plants was found to be accomplished only through wounds. The bacteria were able to produce infection if placed only on the surface of the injuries.

The type of wound apparently made no difference in the kind of overgrowths, but did influence somewhat (1) the length of the incubation period, (2) the percentage of inoculations producing infection, and (3) the size of the reaction. Extremely shallow wounds were found to be poor infection courts.

Wounds on the underground stems of nursery apple trees in the field remained open for infection a relatively short time, averaging about 2 days.

Callus was found ordinarily to be a barrier against the invasion of the hairy-root organism. Shallow wounding of callus only occasionally resulted in infection by the bacteria.

Insects appeared important in producing injuries that led to hairy-root infection. Root-feeding insects have been commonly observed during three growing seasons in the soil about nursery apple trees. During the study of diseased and healthy trees, insects frequently have been seen eating hairy-root and healthy tissue. Infected lateral roots were occasionally observed in positions that could have been reached only by insects. The development of new infections throughout both the first and second growing seasons in Kansas was most easily explained as due to the agency of insects.

Several experiments suggested by the observations on insects indicated a relation of insects to hairy root. The hairy-root bacteria were isolated from white grubs. Isolations from other insects gave negative results. Insect repellents in preliminary trials reduced the amount of hairy-root infection. Insect barriers considerably reduced the amount of hairy root.

The time of the growing season seemed immaterial so far as the entrance of the organism was concerned.

The age of the nursery apple trees in Kansas, where trees are grown only 2 years, did not seem to influence susceptibility to infection by the hairy-root organism.

All sizes of nursery apple trees were found infected in the same field under natural conditions and all sizes became infected when inoculated with the causal organism.

Varietal susceptibility to infection by the hairy-root organism differed widely, ranging from 12 to 100 percent for the 29 varieties studied in 1930, and from 22 to 100 percent for the 37 varieties studied in 1931.

Previous infection of nursery apple trees apparently did not prevent subsequent infection.

The hairy-root organism appeared to begin its relations with the host tissues in the liquid released by the wounds.

The bacteria appeared to be located in the intercellular spaces in both Paris daisy and apple stems.

The presence of the organisms between the cells of the tissues bordering the wounds stimulated a multiplication of the cells in those localities, which resulted in the formation of somewhat circular areas of hyperplasia.

The development of infections without wounds has not been observed on apple trees.

The bacteria were isolated from the surface and subsurface parenchymatous tissues of the enlargements.

The bacteria were found to be abundant on the surface of the hairy-root enlargements.

The bacteria were found often in the soil near the hairy-root formations.

The bacteria overwintered in soil kept either in the field or with nursery stock in the storage cellar. The longevity of the bacteria in field soil which had been steamed or left untreated has been found to exceed 1 year.

The bacteria may be spread long distances by shipments of nursery stock. The seedling root was found to be one medium of transmission of the causal organism from one crop to the next.

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SEASONAL DEVELOPMENT OF HAIRY ROOT, CROWN GALL, AND WOUND OVERGROWTH ON APPLE TREES IN THE NURSERY[1]

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INTRODUCTION

The seasonal development of hairy root, caused by *Phytomonas rhizogenes* Riker et al., crown gall, caused by *Phytomonas tumefaciens* (Smith and Town.) Bergey et al. (synonym, *Bacterium tumefaciens* Smith and Town.), and wound overgrowths on apple trees in the nursery has been studied in relation to certain factors that might influence the initiation, development, and control of these diseases. This work was undertaken as a part of the program on the complex graft-knot problem. After the differentiation of these various diseases from one another, a successful effort was made by the senior writer and his associates (*8, 10*)[3] to induce them at will under controlled conditions. A study was then made by Hildebrand (*4*) of the life history of the hairy-root organism in relation to pathogenesis, with special emphasis on the mechanism of infection. In connection with these lines of work, it appeared desirable to know at what times these overgrowths on nursery apple trees were initiated, under what conditions they developed, and the relative importance of the infections periods.

This study was made in eastern Kansas near the center of the region where piece-root apple grafts are planted in large numbers, where apple seedlings are grown, and where hairy root has occurred with a frequency satisfactory for detailed study. Control measures which had proved adequate in other places often either failed or were only moderately successful in this locality. This fact emphasized the value of the location for studying one of the most difficult phases of control; consequently the analyses presented are of a severe rather than an average situation. The observations and experiments carried out in considerable detail in eastern Kansas were correlated with findings in other nurseries. At least once every fall the results were compared with those secured in Iowa, Minnesota, Missouri, Nebraska, Kansas, Oklahoma, and Wisconsin.

A preliminary statement of some of the earlier phases of this work has already appeared (*11*).

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[2] The writers are indebted to Dr. Sarah L. Doubt, Department of Botany, Washburn College, for laboratory facilities, and to Eugene H. Herrling, Department of Plant Pathology, University of Wisconsin, for preparing the illustrations.

[3] Reference is made by number (italic) to Literature Cited, p. 912.

EXPERIMENTAL PROCEDURE

The procedure followed in these studies is an adaptation of that described by Keitt and Jones (*5*). The methods used were selected in consultation with Dr. Keitt.

ENVIRONMENT

The histories of the fields employed varied somewhat. The field in which experimental grafts were planted in 1929 had carried crops of potatoes in 1926, 1927, and 1928. The field in which experimental grafts were planted in 1930 had previously grown three successive crops of corn. The field which was used for experimental grafts in 1931 had grown corn in 1928 and 1929 and apple seedlings in 1930.

The soil reaction was tested from time to time in the three fields in which experimental grafts were planted. The reaction was approximately pH 5.0 in all the fields. The different tests showed comparatively little variation.

Air temperatures were recorded by a thermograph housed 4 feet above the ground in an instrument shelter located among the first-year experimental trees.

The relative humidity of the air was recorded by a hygrograph corrected by the use of a sling psychrometer (U.S. Weather Bureau pattern) and Marvin's (*6*) psychrometric tables.

Soil temperature at the level of the graft union, between 4 and 5 inches beneath the soil surface, was recorded with a soil thermograph. All the instruments were checked daily.

Soil-moisture samples were taken daily from about 4 inches below the surface. The water in the soil was measured in terms of the moisture-holding capacity. With a technic like that used by Riker (*7*), the moisture-holding capacity of the soil in each of the three experimental fields was found to be approximately 38 percent of the oven-dry weight. The amount of water in the daily samples was measured by Bouyoucos' (*3*) rapid method and calculated in terms of the moisture-holding capacity.

The rainfall for each 24-hour period was measured with a rain gage having a 3-inch cup.

HOST DEVELOPMENT

The plant used for all of these studies was the Yellow Transparent variety of apple. Since the diseases studied occur most frequently at the union, the experimental trees were all grown from piece-root grafts made with Kansas seedlings.

The factors of host development considered were the height of the tree and the diameter near the ground level. Measurements were taken at 2-week intervals on 300 first-season and 300 second-season trees. The same trees were used for successive measurements. The height in inches was measured from the original scion where growth began to the top of the main stem. After the early stages of development had passed, the lower point corresponded rather closely with the soil level and was chosen to avoid variations in measurements due to changes in the soil level incident to cultivation. The diameters of the trees in sixteenths of an inch were measured with a nurseryman's caliper about 1 inch above the point from which the new growth left the original scion. This location on the stem was chosen because it was easy to find, was usually just above the ground level, and was generally the place where the stem had the greatest diameter.

STIMULATION OF OVERGROWTHS

Nonparasitic overgrowths were induced in two ways. In some cases aluminum-alloy wire was wrapped twice about the scion a short distance above the union. In other cases a cut about half-way through the scion was made with an upward pull of the knife so that a wedgelike projection of tissue extended down. Direct healing was prevented by inserting a strip of adhesive tape in the cut. This cut produced a result similar to that often found in a poorly fitted graft.

The parasitic overgrowths were induced by inoculations with pure cultures of the causal bacteria. Crown gall was induced with culture A–1 of *Phytomonas tumefaciens*, which was grown from an individual cell. Hairy root was induced with culture C–1 of *P. rhizogenes*. This culture was also grown from a single cell. An account of the purification of these cultures and of their bacteriological characters has already appeared (*14*).

Inoculations at grafting time were made by smearing the cut surfaces of the scions and roots with a suspension of bacteria before these parts of the graft were fitted together and the union was wrapped.

The experimental blocks of trees consisted of a series of parallel rows, each row of which was used for the study of a certain factor, as explained later. Thus on a given day 10 trees in the first row were inoculated with the crown-gall organism, 10 trees in the second row were inoculated with the hairy-root organism, 10 trees in the third row were inoculated with a mixture of the hairy-root and crown-gall organisms, 10 trees in the fourth row received control wounds, 10 trees in the fifth row were girdled with wire, and 10 trees in the sixth row were cut and taped to stimulate wound overgrowth. On successive weeks during the season similar treatments for the production of the overgrowths were made on adjacent trees under comparable conditions.

The method of inoculation during the growing season was as follows: A trench to the depth of the union was made in the soil about 3 inches away from the trees on one side of the nursery row. The trench was opened this distance away to prevent injury to the main stems of the trees while digging. The soil was removed from around the individual trees with a dibble and by hand. Any particles that remained on the main stem were wiped away. Inoculum from a culture of the bacteria was applied with a cotton swab to the stem surface, usually in five places spaced about 1 inch apart. With a scalpel held at an angle, two thrusts were made through each of the drops of culture deep into the stem. Control wounds were made in a similar manner except that no bacteria were employed. During the 1930 and 1931 seasons strips of adhesive tape were applied over both the inoculated and the control wounds to reduce chance contamination from the soil and to keep out insects. Promptly after inoculation the soil was thrown back into the trench so as to cover the topmost wound by about 2 inches.

DEVELOPMENT OF ARTIFICIALLY INDUCED OVERGROWTHS

After the inoculations attention was given to the incubation periods necessary for the development of distinct symptoms.

The incubation period for crown gall was determined by making inoculations at weekly intervals throughout the season with a single-

cell culture, A–1, of the crown-gall organism. In 1929 the first inoculations were made, as described earlier, on May 15 and the last on September 11. The corresponding first and last inoculation dates for 1930 were May 12 and September 8, respectively, and for 1931, May 11 and September 14. Because of the similarity in appearance of callus, crown gall, and hairy root, especially in the early stages, the inoculations were not considered positive until the crown galls had a radial extension of 4 mm. This is in accord with the criteria used by Riker, Hildebrand, and Ivanoff (*10*). The incubation time given in the charts (figs. 1, 2, and 3) for any one period is the average time required for the positive reactions from 50 inoculations. Minimum and maximum times are recorded in figure 4. Approximately 55 percent of the inoculations gave positive reactions.

The development of crown gall after inoculation was noted at weekly intervals for the first 6 weeks, during the early disease stages, and at biweekly intervals thereafter. The number, character, and radial extension of the overgrowths were recorded at successive intervals during the season.

The incubation period of hairy root was determined after making inoculations with a culture (C–1) grown from a single cell of the hairy-root organism. The methods and intervals of inoculation were the same as for crown gall. The incubation period for hairy root was recorded in two ways: (1) The time required for a radial extension of 3 mm and for the appearance of root primordia is indicated on the charts by a small circle in the line indicating the incubation period, and (2) the time required for the development of roots 1 cm long is shown at the end of the incubation period. The incubation time given for any one period is the average time required for the positive reactions from 50 inoculations. The minimum and maximum times are recorded in figure 4. Approximately 70 percent of the inoculations gave positive results.

Similar studies were made of the incubation periods and development of the reactions when mixtures of crown-gall and hairy-root bacteria were used for the inoculum.

The host reactions to uninoculated control wounds made in parallel series were examined as controls on the hairy-root and crown-gall series. The procedure was the same except that no bacteria were employed.

The host reactions to wire girdles and to knife cuts with tape inserts were followed in parallel series and at similar time intervals.

The development of disease from inoculations with the hairy-root bacteria at grafting time of 300 string-wrapped and 300 tape-wrapped grafts was observed at monthly intervals during 1930 and 1931.

NATURAL OCCURRENCE OF OVERGROWTHS

The natural occurrence of hairy root was determined in 1929 through biweekly examinations of the unions of 300 trees grown from string-wrapped grafts. In 1930 and 1931 monthly examinations were made of the unions on 1,200 trees, including 300 first-year and 300 second-year trees grown from string-wrapped grafts, and 300 first-year and 300 second-year trees grown from tape-wrapped grafts.

Observations were made at stated intervals by removing the soil from about the union in the manner previously explained. If a tree was accidentally injured in the process, this fact was noted and data

were taken accordingly. At the end of the experiments comparisons were made between the percentages of overgrowths on trees examined at various intervals and on those left undisturbed. The variations ranged from 0 to 10 percent. Consequently, it appears that injuries incident to the periodic examinations introduced little if any error into the results secured.

INSECT RELATIONS

Insect surveys were made of the soil in which the experimental trees were growing. In 1929 the work was begun intensively on May 5 and carried on until July 12, when it was discontinued. Subsequently only insects that appeared in connection with the other work were collected and preserved. However, the survey was carried through the season in 1930 and 1931. At biweekly intervals three areas of soil 9 inches square and 6 inches deep were taken at random from around three first-year trees. These samples were mixed and one third of the mixture was taken to the laboratory for examination. All insects observed while the larger sample was being divided were also taken. The soil was minutely gone over by spreading it on light brown paper. The insects present were collected and placed in vials containing 70-percent alcohol, for later identification.[4] The observations in 1930 were begun May 29 and finished September 18. The first examination in 1931 was on May 11 and the last on September 29.

EXPERIMENTAL RESULTS

The more significant aspects of the results obtained are considered under the following heads: (1) Seasonal variations, (2) incubation periods, (3) appearance of overgrowths at different stages of development, and (4) date of natural infection. The records are given in figures 1 to 5, inclusive.

SEASONAL VARIATIONS

Seasonal variations were large during the 3 years covered by these studies. These variations provided opportunities for observing the effect of seasonal changes both at different times in the same season and at corresponding dates in different seasons (figs. 1, 2, and 3). Since the details are recorded in the figures, only the grosser aspects are discussed here.

The air temperatures during 1929 at Topeka, Kans., were generally below those of the average year in that locality. On the other hand, the air temperatures during the season of 1930 were generally above normal, favorable growing temperatures extending into late October. Although the season of 1931 was considerably warmer in June than the previous two seasons, it was about normal in July, below normal in August, and considerably above normal in September.

The average air-humidity readings for the season of 1929 were much higher than those of the two subsequent seasons. During 1930 the average air humidity was the lowest of the three seasons. But for the month of June the 1931 season had an average air humidity falling between those of the preceding two seasons.

The soil temperatures at the level of the graft unions quite naturally followed the trends of the air temperatures. During the day the soil temperatures lagged behind the air temperatures, but at night the

[4] The identifications were made by C. L. Fluke, Jr., and E. M. Searls, of the University of Wisconsin.

opposite relation occurred. As was expected, the extremes of soil temperature were much closer together than those of air temperature.

Soil-moisture data were closely correlated with those of soil temperature and rainfall. During 1929, because of the relatively low soil temperature and high average rainfall, the soil-moisture values were generally the highest of the three seasons. In 1930, because of relatively high soil temperature and low average rainfall, the average soil humidity was the lowest. High soil temperature in June 1931 was accompanied by low soil moisture, largely, perhaps, because of the extremely low rainfall during that month. In September, because of relatively high soil temperature and relatively heavy rainfall, the soil moisture ran a middle course.

Rainfall was rather variable during the three seasons. It was above normal in 1929 and below normal in 1930. In 1931 it was below normal in May and June and approximately normal the remainder of the season.

The host development of nursery apple trees for the 1929 season, according to the local nurserymen, was the largest of any season over a period of 40 years, despite the fact that the trees went into dormancy earlier than in the two following seasons. The large growth was accomplished under conditions of higher soil moisture and somewhat lower temperature than usually obtained. There was no slack period due to soil moisture or temperature conditions. The growth of the trees in 1930 suffered because of the drought and abnormally high temperatures in July and August. However, considerable growth was made during September and October, which partially offset the less favorable period. In 1931 the less favorable period for host growth was in June, when abnormally high temperatures and low soil moistures prevailed. Because of a well-distributed rainfall throughout the remainder of the season the trees overcame much of the handicap of July and August and approached the large growth of the 1929 season. The high temperatures and normal moisture conditions of late fall enabled the first-year trees to make more than average growth, and the second-year trees to make the largest growth of the three seasons. The height and caliper measurements were closely correlated during the three seasons, with one exception. The early fall of 1929 caused the trees to stop growth in height in late September, but the growth in diameter continued into October. Records of the growth of the trees are given in figures 1, 2, and 3.

Insect surveys showed that a number of different kinds of root-chewing insects were present during the growing seasons of 1929, 1930, and 1931. Since the larvae only were found in most instances, it was not possible always to determine the species or even the genus. According to information received from C. L. Fluke, Jr., and E. M. Searls, of the University of Wisconsin, the insects considered most important from the standpoint of opening infection courts were the white grubs (Phyllophaga), wireworms (Elateridae), and fungus gnats (Mycetophilidae). White grubs and wireworms were continually present, but were more abundant in June, early July, and September than in May and August. Fungus gnats were more commonly associated with crown gall and were less generally present than the white grubs or wireworms. From these studies it appears that various insects capable of opening infection courts for crown-gall or hairy-root bacteria were present in varying abundance throughout

the growing seasons. The relation of insects to crown-gall infection on raspberries has been discussed by Banfield (*1*, *2*). A similar relation to hairy-root infection on apple trees has been investigated by Hildebrand (*4*).

INCUBATION PERIODS

The time required for the development of excess callus and wound overgrowth following wire girdling or cuts was 2 weeks or more, depending upon the rapidity of the growth of the plant.

Crown-Gall Organism

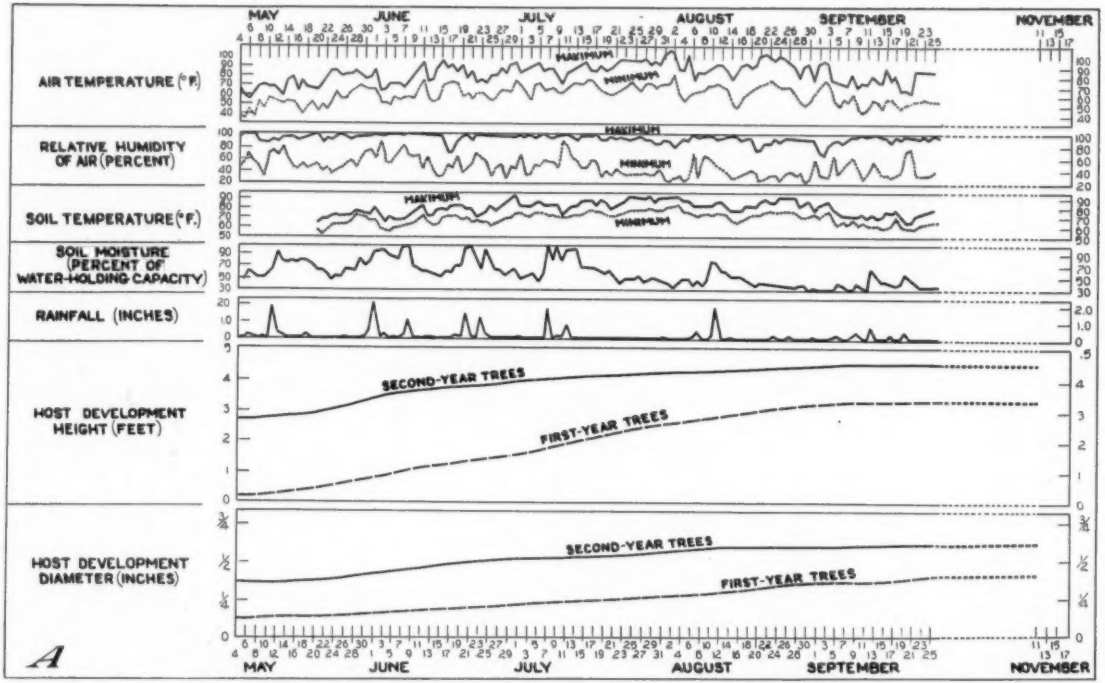
The incubation periods of the crown-gall organism were on an average much longer in the earlier and later parts of the season than in the middle. An inoculation was considered positive only when the gall had a radial extension of 4 mm after 3 weeks (fig. 8, *F*). The minimum and maximum periods are given in figure 4. Doubtless larger numbers would have produced more regular curves. Charts giving the average incubation periods (figs. 1, 2, and 3) show what appears to be a rather close correlation between short incubation periods and warm weather. Only slight differences were noticed in the length of the incubation periods for corresponding weeks in the different growing seasons.

These incubation periods are concerned only with vigorously growing trees. Inoculations on trees which were making little or no growth did not induce disease until the trees began to develop. Inoculations on such trees were often negative, but reactions have sometimes appeared after normal incubation from the time growth began. Thus after inoculations made late in the season overgrowths might not appear until the following spring. The incubation periods on trees growing under favorable conditions had a definite maximum period after which no infection appeared and which seemed to be correlated with the rapidity in growth of the tree and with temperature.

Hairy-Root Organism

The incubation periods for the hairy-root organism were followed in the same way as for the crown-gall organism. The averages are shown in figures 1, 2, and 3, and the minimum and maximum periods in figure 4. The average length of time necessary for development of roots 1 cm long ranged from approximately 3 weeks in the warm period of the growing season to 9 or more weeks in the cool periods at the beginning and end of the season. Two stages in the incubation period of hairy root were observed. The first stage, indicated by a circle on the charts (figs. 1 to 3), was reached when the average hairy-root enlargement had a lateral extension of approximately 3 mm and when root primordia were beginning to show (fig. 6, *A*, after 3 weeks). The second stage in the incubation period was reached when the hairy roots had a lateral extension of 1 cm (fig. 6, *A*, after 5 weeks). As in the case of crown gall, only slight differences were noticed in the length of the corresponding hairy-root incubation periods in the different seasons.

Experiments were conducted to determine when disease would develop from inoculations made at the time of grafting. These trials were made in 1930 and 1931 on both string-wrapped and tape-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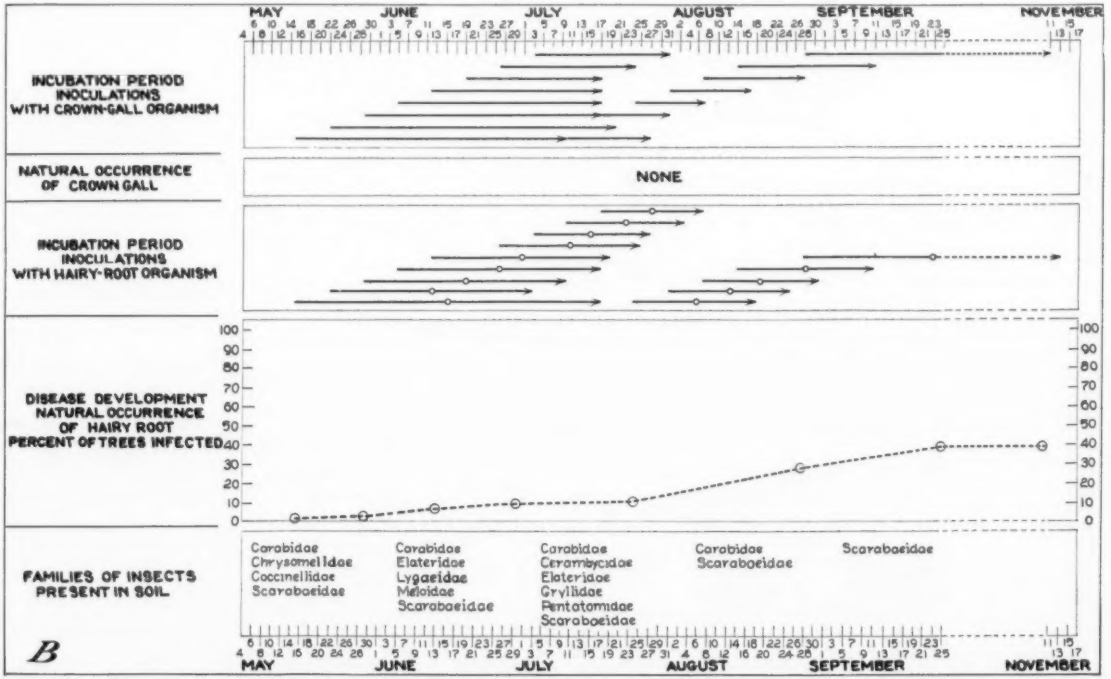
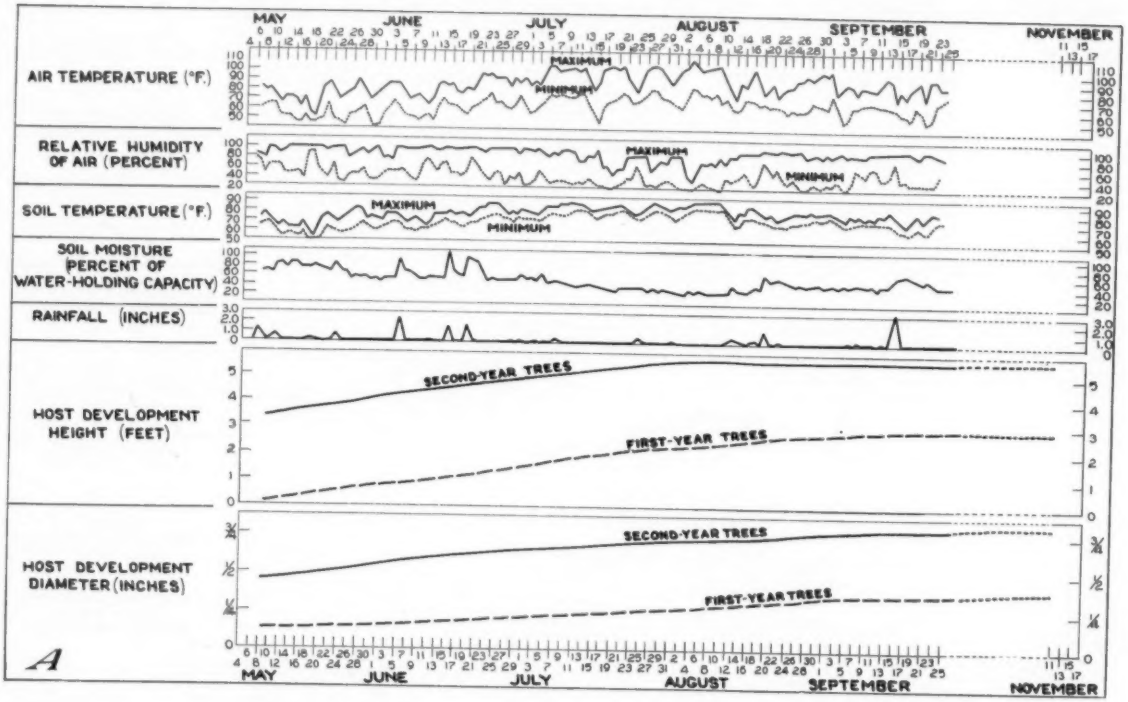


FIGURE 1.—Summary of data relating to seasonal development in 1929 of hairy root and crown gall on nursery apple trees grown from string-wrapped piece-root grafts: *A*, Environmental conditions and tree growth; *B*, incubation periods, natural occurrence of disease, insects present, and seasonal development of hairy root and crown gall.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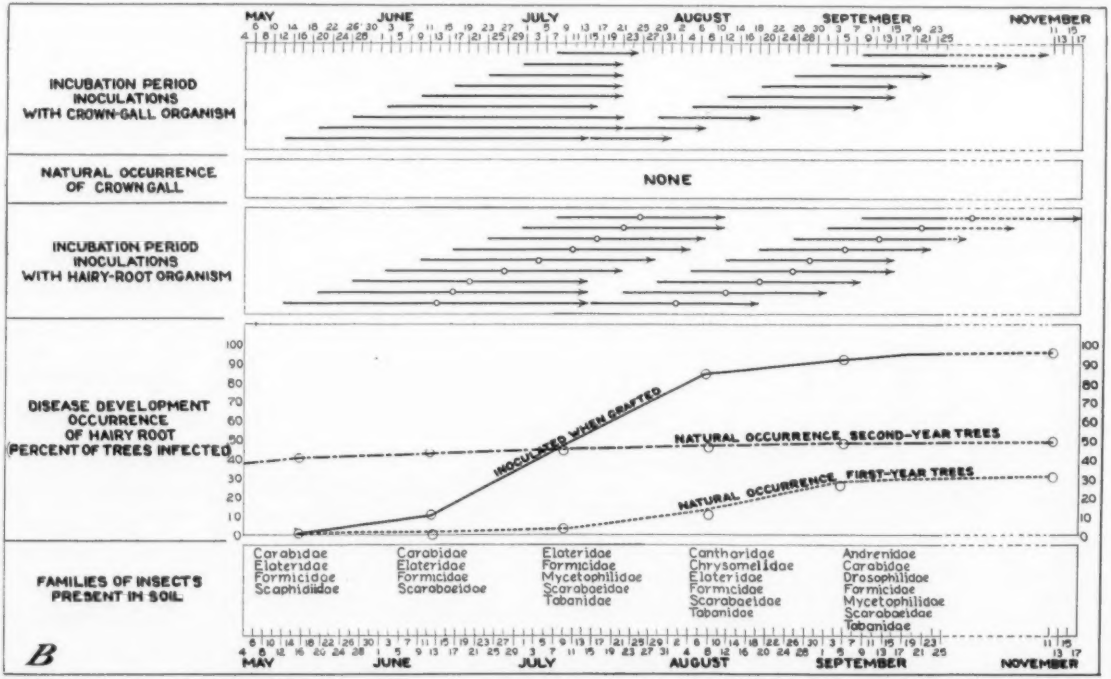
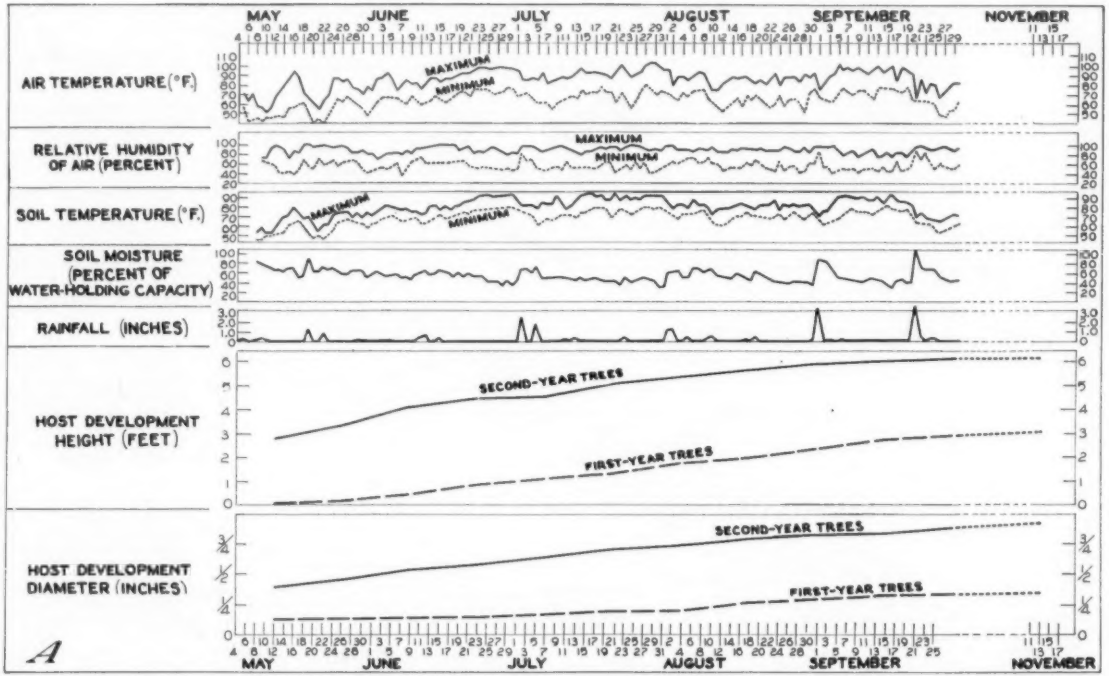


FIGURE 2.—Summary of data relating to seasonal development in 1930 of hairy root and crown gall on nursery apple trees grown from string-wrapped piece-root grafts: *A*, Environmental conditions and tree growth; *B*, incubation periods, natural occurrence of disease, insects present, and seasonal development of hairy root and crown gall.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HOST DEVELOPMENT HEIGHT (FEET)
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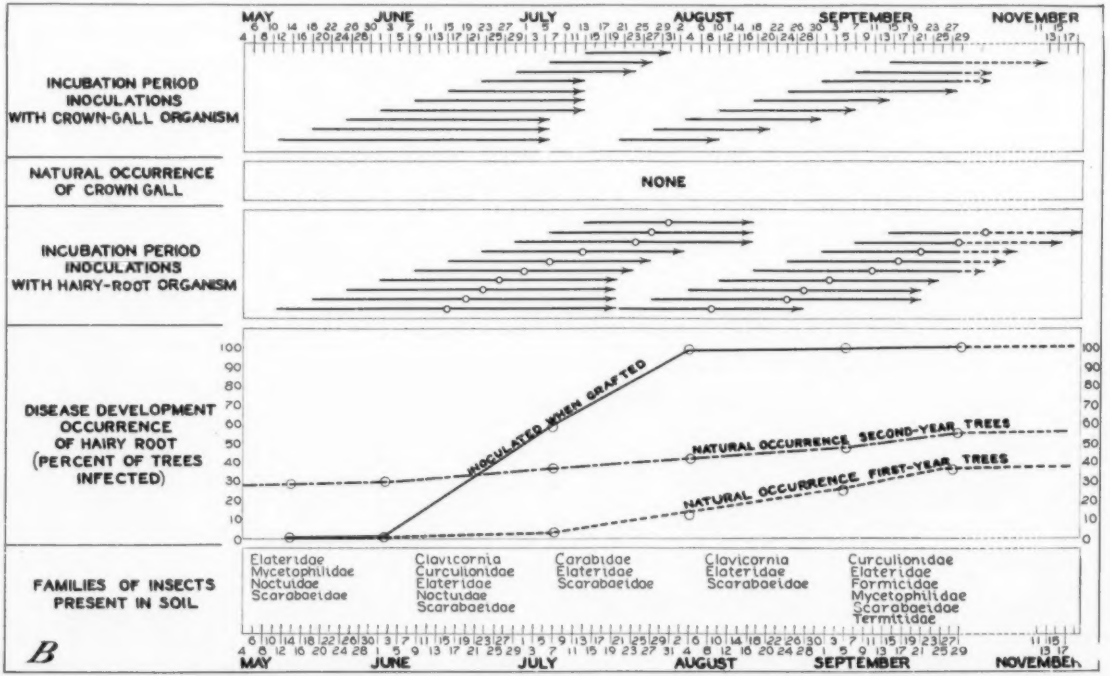

FIGURE 3.—Summary of data relating to seasonal development in 1931 of hairy root and crown gall on nursery apple trees grown from string-wrapped piece-root grafts: *A*, Environmental conditions and tree growth; *B*, incubation periods, natural occurrence of disease, insects present, and seasonal development of hairy root and crown gall.

wrapped grafts. The results are recorded in figures 2, 3, and 5. The average time at which hairy-root symptoms (i.e. roots at least 1 cm long) developed in the string-wrapped grafts appeared to be the early part of July in both 1930 and 1931. The minimum was early in

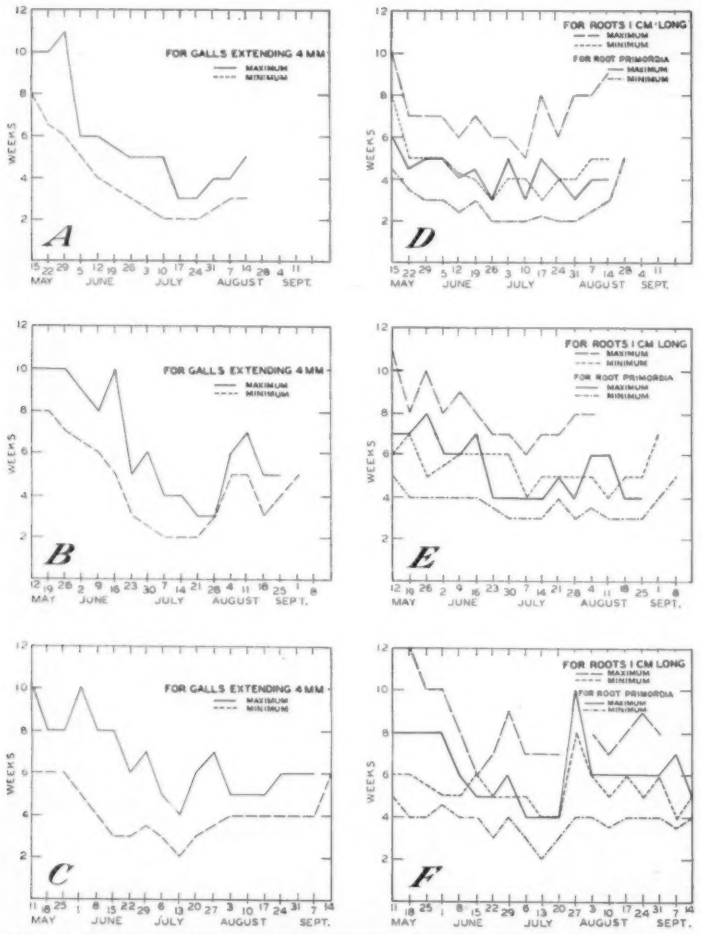

FIGURE 4.—Progressive variations in the length of incubation periods following inoculations made, on the dates indicated, with crown-gall and hairy-root bacteria during the active growing seasons of 1929, 1930, and 1931. The characters of the overgrowths indicating a positive reaction are explained in the text: *A*, Crown gall, 1929; *B*, crown gall, 1930; *C*, crown gall, 1931; *D*, hairy root, 1929; *E*, hairy root, 1930; *F*, hairy root, 1931.

June. The maximum was difficult to determine because of the occurrence of natural infection, which complicated the results. Most of the grafts showed infection by early August. As judged from other inoculations, the maximum probably did not greatly exceed this.

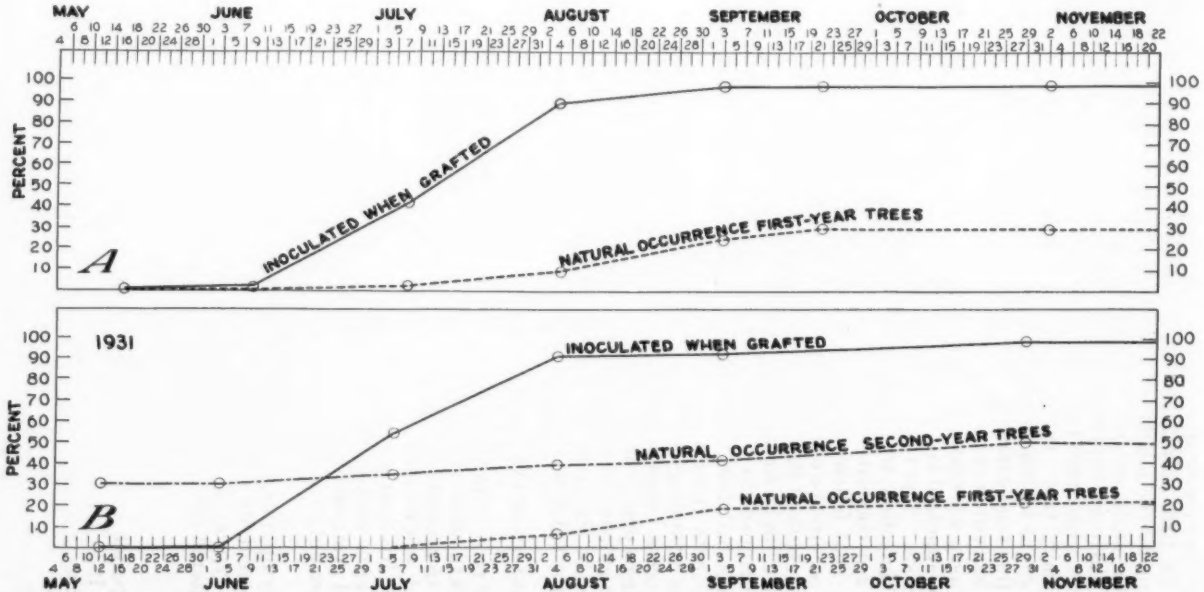

FIGURE 5.—Summary of data relating to hairy-root development in 1930 (*A*) and 1931 (*B*) on nursery apple trees grown from tape-wrapped piece-root grafts.

The occurrence of hairy-root symptoms, following inoculations at the time the tape-wrapped grafts were made, was studied in parallel series. In 1930 only 2 percent of the inoculated grafts had shown symptoms by the middle of June. Hairy root was noted on 41 percent of the grafts on July 9, on 90 percent early in August, and on 97 percent early in September. In 1931 none of the grafts showed symptoms when examined early in June. Early in July, 54 percent showed hairy root; early in August there was 91 percent; and by the end of the season there was 99 percent. Like the string-wrapped grafts, the tape-wrapped grafts that were inoculated when made showed hairy-root symptoms early, during the season of long incubation periods, whereas symptoms of hairy root resulting from natural infection appeared later, during the season of relatively short incubation periods. The tape wrapping had little if any influence in preventing infection when inoculations were made at grafting time. Little if any correlation was noted between the time of the occurrence of hairy root following inoculation at grafting and the time of the natural occurrence of the disease.

The strength of the tape wrappers was found to diminish gradually, until the cloth broke under the slightest strain. Where the wrapping was overlapped the cloth lasted much longer. Frequently the tape wrappings had lost their strength by June, so that the hairy-root developments from inoculations easily came through. Sooner or later the wrappings were cracked open by the growth of the trees. This process began in June and progressed with varying rapidity, depending upon such factors as the moisture and temperature of the soil, the amount of overlapping of the wrapper, and the increase in diameter of the tree. It is clear that while the wrapper remained intact, the union was protected against root-chewing insects.

APPEARANCE OF OVERGROWTHS AT DIFFERENT STAGES OF DEVELOPMENT

Hairy root, crown gall, mixtures of these two, and callus or wound overgrowth were studied as they developed after inoculations or special treatments made at different times. The results of these extended studies conform in general with those obtained in limited trials by Riker et al. (*9*). Since a complete new series of tests was started each week during three active growing seasons, the volume of material available was large. Consequently the development of only one representative series for each type of overgrowth is considered here.

The symptoms of the different enlargements in the various stages of development are illustrated in figures 6 to 8. The descriptions given are of typical symptoms that followed the different treatments administered during the latter part of May.

Hairy Root

Hairy-root development is described first for one of the series made during 1930. The cultures and methods of inoculation employed have already been given. The development of the various symptoms was more rapid on the Yellow Transparent trees in Kansas than on Wealthy trees in Wisconsin (*9*).

After 1 week the scalpel cuts in which the inoculations were made showed practically no change from the control scalpel cuts. A slight

FIGURE 6.—Hairy-root symptoms after wound inoculations, as they appeared during the first year on scion wood below ground: *A*, After 1, 2, 3, 4, 5, and 6 weeks, respectively, from left to right; *B*, after 8, 10, and 12 weeks; *C*, after 16 and 18 weeks; *D*, after 24 weeks.

formation of wound tissue was apparent, especially about the exposed portions of cambium.

After 2 weeks the frosty calluslike tissue had so increased in size that it practically covered the injury. Small hemispherical frosty knobs of new tissue appeared sometimes to extend from the callus. No difference was noticed between the inoculated and control wounds. The lateral extension of the tissue was approximately 2mm.

After 3 weeks the hairy-root tissue had grown until it had an average lateral extension of about 3mm. This was somewhat greater than

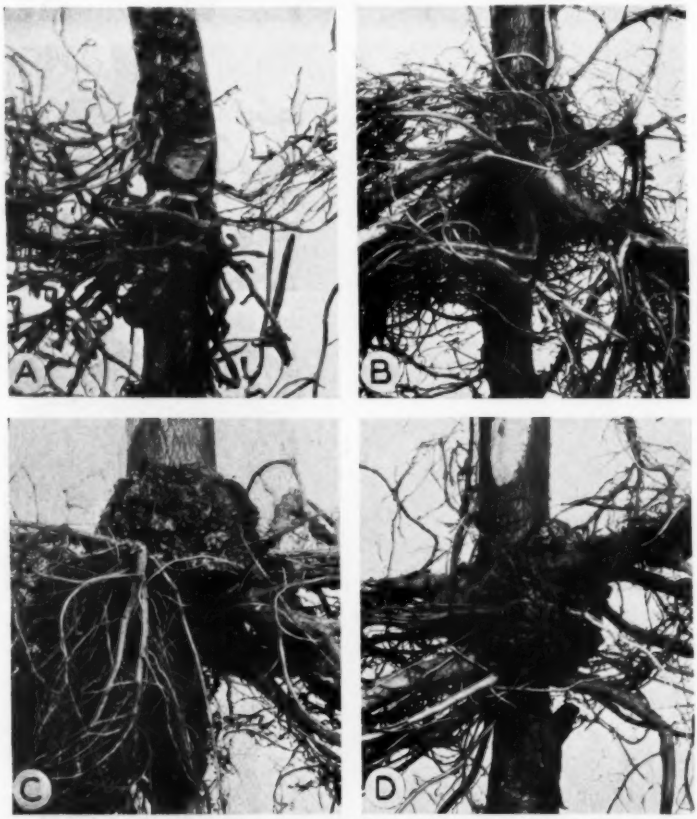

FIGURE 7.—Hairy-root symptoms after wound inoculations, as they appeared during the second year on scion wood below ground: *A*, In May. Many of the smaller roots were killed by frost.; *B*, In August. Certain of the roots appear much larger than others. *C* and *D*, In October. Wound-overgrowth tissue appears in conjunction with that of hairy root.

the callus at the control wounds. At this time small, more or less round protuberances of tissue, root primordia, approximately 2 mm in diameter, made their appearance from the surface of the basal tissue. Often, but not always, these appeared to be further developments of the knobs mentioned earlier. This stage is indicated by circles in the arrows of the charts designating incubation periods (figs. 1, 2, and 3).

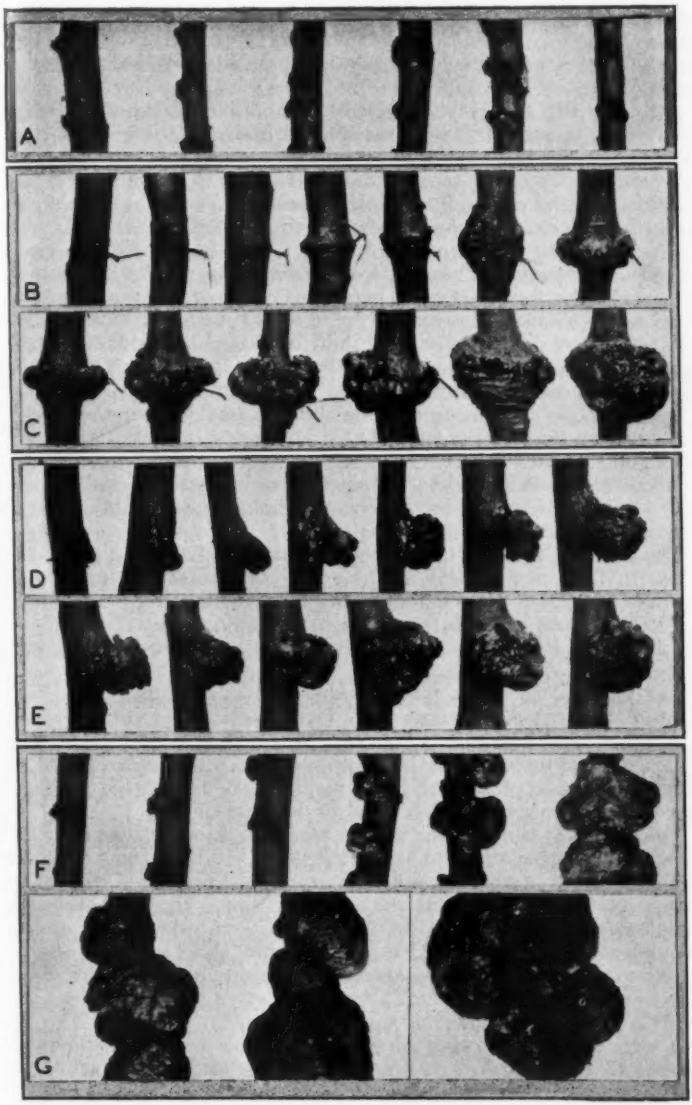

FIGURE 8.—Several types of overgrowths after various treatments, as they appeared during the first year on scion wood below ground: *A*, Wounds like those used for inoculation, except that no bacteria were employed, after 1, 2, 3, 4, 5, and 6 weeks, respectively, from left to right; *B*, induced by wire girdles, after 1, 2, 3, 4, 5, 6, and 8 weeks, respectively, from left to right, and (*C*) after 10, 12, 14, 16, 18, and 20 weeks; *D*, induced by knife cuts, into which were inserted bits of adhesive tape, after 1, 2, 3, 4, 5, 6, and 8 weeks, respectively, from left to right, and (*E*) after 10, 12, 14, 16, 18, and 20 weeks; *F*, crown galls that followed wound inoculations as they appeared after 1, 2, 3, 4, 5, and 6 weeks, respectively, from left to right, and (*G*) after 8, 10, and 12 weeks.

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After 4 weeks both the basal tissue and the protuberances had enlarged until the average lateral extension was approximately 4 mm. Some of the protuberances had taken on more definitely the appearance of root primordia. In exceptional cases small fleshy roots were starting.

After 5 weeks the basal enlargements had increased a little and root primordia were quite common. Small fleshy roots were also more frequent. The root primordia and roots gave the surface a rough and irregular appearance, in contrast with the relatively smooth surface of crown galls. Some of the small roots were over 1 cm long, indicating the final stage of the incubation period.

After 6 weeks the number of fleshy roots was decidedly greater, and the older ones had reached a length of 2 cm. After this time the basal enlargements increased in size very slowly and some became more or less completely covered by the root developments.

After 8 weeks the fleshy roots had increased considerably both in number and in length. Many had begun to branch, and a few small fibrous roots were found.

After longer periods during the first season following inoculation there was a progressive increase in the number and length of the hairy roots. Large masses of both fleshy and fibrous roots were very common at the end of the growing season. The basal enlargements were ordinarily rather inconspicuous the first year and were well covered with root developments.

The amount of moisture in the soil appeared to be an important factor in the development of the roots. Inoculations made aboveground or in the upper portions of the soil, where it frequently became dried out, showed little or no root development. Evidence was frequently found that roots had started but had been killed by subsequent drying of the surface soil.

The vigor of the trees likewise appeared to influence the development of the hairy-root symptoms. Grafts which had not become well established and had not produced new growth at least a foot high made little or no response to hairy-root inoculations. The relation between the size of tree and the incidence of hairy root at digging time has been more fully discussed by Hildebrand (4).

The period in the growing season when the inoculations were made likewise influenced the rapidity with which the symptoms developed. The incubation periods for different stages of the disease were shorter during the summer than in the spring or fall. In some cases when inoculations were made in September, no symptoms were observed until the following year. Although the time at which they appeared varied considerably, the sequence of symptoms remained the same.

During later development after inoculation the hairy-root overgrowths showed a somewhat different character (fig. 7). The roots that were actively growing when cold weather set in and the ground became frozen were killed. However, those that had reached sufficient maturity were able to withstand the winter and to continue their activity the second year. By June of the second year, certain of the larger roots had grown considerably, both in length and diameter. Many small lateral roots appeared that were more likely to be fibrous than fleshy. However, fleshy roots from the basal tissue were quite common.

The basal enlargements likewise had changed somewhat in character. They had grown considerably and had taken on the rather characteristic deeply convoluted type of growth. These various convolutions were built up for several layers and not infrequently particles of soil were incorporated for some distance within the tissue. This type of growth is illustrated in figure 7.

By the middle and latter parts of the second season both the basal enlargements and the hairy roots had increased markedly in size. In all the specimens examined the large lateral roots which developed as part of the hairy-root overgrowth seemed to have established definite connection with the main stem and to be functioning in a somewhat normal capacity.

Nonparasitic tissue of the wound-overgrowth type not infrequently appeared in association with the basal enlargements of the hairy-root development. This seemed to occur in part as a reaction to the interruption in the downward flow of elaborated food in the stem. Apparently the hairy-root development had something of a wounding or girdling action upon the stem, which resulted in the formation of wound-overgrowth tissue (fig. 8) in combination with the hairy-root tissue. All different stages of combination between wound overgrowth and hairy root seemed to occur. Doubtless in some cases the wound-overgrowth tissue occurred as a result of the girdling action of the hairy-root tissue, whereas in other cases formations which were wound overgrowths at the beginning became infected and hairy-root tissue subsequently developed.

Crown Gall

Studies of crown gall were made similar to those of hairy root. After 2 weeks the reactions to inoculations were similar to those secured from the inoculations with the hairy-root organism. Frosty hemispherical knobs appeared similar to those of hairy root. However, the knobs on crown gall were not observed to develop further. After 3 weeks the lateral extension of the crown gall was about 1 mm greater than that of hairy root and the surface was lobed and comparatively smooth. After 4 weeks the crown galls had increased considerably in size and often showed a lateral extension of 5 to 8 mm. No root primordia were observed.

After longer periods the basic characters of the crown galls did not change, although the formations increased greatly in size as the weeks passed. No roots have been found growing directly from the crown-gall tissue and only infrequently from the main stems of the Yellow Transparent apples near the gall tissue. The sequence of development of the crown galls is shown in figure 8, *F*, *G*. The influence of temperature and moisture on the development of crown gall under controlled conditions has been reported by Riker (*7*).

Mixtures of Crown Gall and Hairy Root

Parallel inoculations were made with mixtures of the crown-gall and hairy-root bacteria. The resulting overgrowths developed along the lines previously mentioned for crown gall and hairy root and showed all gradations between those two types of formations. The time of the growing season seemed to exert some influence in determining which type of development would appear first and predominate. The ascendancy of the hairy-root organism was greatest com-

paratively early or late in the growing season, when the temperature was somewhat low. On the other hand, in approximately the middle of the growing season, when the temperature was somewhat high, the crown-gall organism appeared to have the advantage.

Wound and Callus Overgrowths

The control wounds that were made in conjunction with the inoculations for crown gall and hairy root produced only slight reactions. For the first 2 weeks there was practically no difference between the control injuries and those inoculated with the two organisms (fig. 8). However, after 3 weeks the callus formations which appeared began to decrease in size as the process of healing progressed; by the fourth week the soft outer tissue had begun to slough off; after 5 or 6 weeks the characteristic wound tissue had formed and the development of these wound reactions ceased. Natural infection of the control wounds was rare.

Parallel studies were made upon wound overgrowths induced by girdling with wire. As already stated, aluminum-alloy wire was wrapped twice around the stem and then fastened in order to determine what the reaction of the host plant would be to this interruption in the downward passage of elaborated food. After 1 week there was practically no change, but after 2 weeks the wire wrapping was very tight about the bark (fig. 8, *B*, *C*). After 3 weeks it seemed to be cutting into the bark tissue. After 4 weeks a swelling appeared above the wire that in some cases was sufficient almost to hide it from view. After 5 weeks this wound tissue had increased to a lateral extension of 2 to 4 mm and the wire was almost completely hidden. After 6 weeks the growth had increased in size and after 8 weeks it had a lateral extension of approximately 7 mm around most of the stem. These nonparasitic overgrowth developments so increased in size as the season progressed that at the end of the growing season their diameter was several times that of the main stem. The surface of this tissue had more or less broad undulations very different from the comparatively smooth lobes of the crown-gall tissue or the deep convolutions of the hairy-root tissue. There was a definite cortex. This type of tissue resembled more closely the tissue of hairy root than that of crown gall. As already stated, complete intergradations were found between the surface characters of this type of development and those of hairy root.

Similar wound overgrowths developed after certain knife injuries made on the underground parts of apple stems, about an inch above the union, by an upward cut through one third to one half of the diameter of the stem. A small piece of adhesive tape was inserted under the tissue thus cut in order to prevent its direct healing and to simulate conditions at the lower ends of scions that failed at the tips to make union with the roots. Trees without the tape barriers in the cut usually healed normally in a few weeks without the development of excessive callus. In trees having tape barriers, a small callus had formed on the lower tip of the cut tissue after 1 week (fig. 8, *D*). Within 2 weeks the callus had grown considerably and after 3 weeks it had a lateral extension of several millimeters. The increase in size continued until characters of wound overgrowth appeared similar to those induced by wire girdles. At the end of the season overgrowths of this type had a lateral extension from 1 to 3 times greater than the

diameter of the main stem and showed the characters of wound overgrowth as already described.

These studies on various overgrowths of known origin were very helpful in the diagnoses of overgrowths that developed under natural conditions.

DATE OF NATURAL INFECTION

The natural occurrence of the various overgrowths in this graft-knot complex was recorded at stated intervals throughout three growing seasons. No natural occurrence of crown gall was found among the trees under observation. In other plantings in this locality where a large number of trees were observed, approximately 0.1 percent of the trees were affected with crown gall. The early stages of excess callus or wound overgrowths appeared from time to time. Some of these were grown over as the trees developed (*12*), and some, like the girdle or wound knots (fig. 8), continued to develop; but in this particular locality most of them sooner or later became infected by hairy-root bacteria, and their classification was changed to hairy root. Hildebrand (*4*) has recorded the activity of root-chewing insects in opening infection courts in such tissue. Since the location of this work was made largely on the basis of the severity of the hairy-root infection, this result was expected.

The natural occurrence of hairy root in 1929 on first-year apple trees grown from string-wrapped grafts was recorded at weekly intervals (fig. 1). Natural infection appeared slowly until the end of June, when 11 percent of the trees showed symptoms of disease. By the end of July practically no increase had occurred. However, by the end of August, 28 percent of the trees showed infection; and by the end of September, 38 percent. After this time, apparently correlated with lower temperature and perhaps cessation of growth by the host plant, there was comparatively little increase until the return of warm weather. During the second year (1930), on the same trees (fig. 2), there was a progressive increase up to 49 percent in November, when the trees were removed.

In 1930 the natural occurrence of hairy root on first-year trees (fig. 2) grown from string-wrapped grafts was quite similar to that in 1929. There was 4 percent infection early in July, 13 percent early in August, and 27 percent early in September. There was relatively little increase between that time and the middle of November, when there was 30 percent. During the following year (1931; fig. 3) on the same trees, that were second-year trees by this time, there was practically no development until June. By early July there was an increase to 37 percent, by early August to 42 percent, by early September to 48 percent, and by late October to 57 percent.

In 1931 the natural occurrence of hairy root on first-year trees (fig. 3) grown from string-wrapped grafts was very similar to that of the previous year. Only 3 percent appeared early in July. Early in August there was 14 percent; early in September, 26 percent; and late in October, 38 percent.

Similar results were secured for tape-wrapped grafts (fig. 5). However, the appearance of symptoms was delayed somewhat by the tape wrapping.

The natural occurrence of hairy root in 1930 on trees grown from tape-wrapped grafts was recorded as before. The first hairy-root symptoms appeared early in July, when 2 percent of the trees showed

hairy root. Early in August, 9 percent showed hairy root; early in September, 25 percent; and late in September, 30 percent. The next year (1931) the number of trees infected rose gradually to 50 percent.

In 1931 the natural occurrence of hairy root on tape-wrapped grafts began with 6 percent early in August. Early in September there was 18 percent, and late in October 21 percent.

The date of natural occurrence of most of the knots on tape-wrapped grafts appeared to be correlated with that of knots on string-wrapped grafts. The date of natural occurrence of knots on both types of grafts seemed correlated with warm weather, active growth of the nursery trees, short incubation periods, decreased protection of the unions by wrappers, and insect activity.

The average date of natural occurrence of an infection appearing on any particular date may be determined by subtracting the number of days of the average incubation period from the date of appearance. For example, to determine the average date of natural infection for new developments that appeared in first-season trees on September 3, 1930, one observes (fig. 2) that the average incubation period ending September 1 began July 21. Therefore, the new infection showing September 3 came from infections taking place about July 21.

The foregoing data indicate that in the plantings under observation most of the natural occurrence of hairy root was not the result of infection induced at grafting time, but was due to certain factors that began to function usually in June after the grafts were planted.

DISCUSSION

The evidence presented in the preceding pages has a definite bearing upon the consideration of control measures. The studies were made in a place where infectious hairy root was prevalent and consequently deal with severe rather than with average conditions. Since nearly all the bacterial overgrowths on the underground parts of nursery apple trees occur at the union, it has been commonly considered that infection takes place approximately at the time the grafts are made. Various antiseptics are therefore applied to the seedlings and to the grafts. Such treatments by the writers have been relatively unsuccessful in this region. Wrappers of adhesive tape have been less efficient for control here than in most places. Apparently, therefore, the chances of developing control methods would be enhanced by determining the time and the conditions under which natural infection takes place.

From the data presented in figures 1 to 5, only a small part of the hairy-root infection naturally occurring appeared to be initiated at grafting time. However, this small part may be important as a potential source of inoculum for subsequent infections.

The date of natural infection for most of the hairy root was apparently correlated with the occurrence of warm weather, rapid growth of the trees, activity of soil insects, relatively short incubation periods, and reduced protection from the wrappers employed. For first-year trees this date was during the middle and latter part of the growing season. New infections continued to occur during the second growing season. In other regions, where the trees stood longer in the nursery row and where hairy root was serious, new infections appeared in the third and fourth seasons (*12*).

The possibility of very long incubation periods perhaps needs further consideration. Relatively dormant trees have been found to carry the hairy-root bacteria for long periods and to show no symptoms until active growth was resumed. The problem to be considered is whether small amounts of inoculum at grafting time might remain dormant long enough to account for the occurrence of hairy root late in the first season and perhaps in the second, third, and fourth season. Although this possibility cannot be easily disproved, the following lines of evidence show that if such a condition occurs it is very unusual: (1) Siegler and Piper (*13*) have reported that bacteria inoculated into aerial parts of trees could be reisolated rarely if at all after 65 days; (2) most of the inoculations made at grafting time have yielded results within ordinary incubation periods and the few infections that appeared later were probably caused by natural infection rather than by the inoculations; (3) the studies on incubation periods showed definite maxima beyond which no symptoms appeared; (4) dormant trees that harbored the bacteria after inoculation in the fall showed symptoms promptly, if at all, when active growth was resumed; (5) the phenomena observed are easily explained by other well-defined factors. It appears, therefore, that the evidence at present available does not justify consideration of common incubation periods as continuing long enough after the date of grafting to account for the date on which the greatest part of the natural infection appeared.

Another phase of the complex hairy-root problem which has been at least partly explained is the failure in this locality of the writers' antiseptic treatments and the lower efficiency of adhesive-tape wrappers. Since root-chewing insects seem implicated during the growing season as important factors in opening infection courts, doubtless the present control measures against graft knots may be supplemented by others that take into account the newly determined date of the natural occurrence of infection.

SUMMARY

The seasonal development of crown gall, hairy root, and wound overgrowth has been followed through the growing seasons of 1929, 1930, and 1931 in Kansas. Crown gall and hairy root were induced by inoculation with single-cell cultures of the causal organisms. Wound overgrowth was induced by wire girdles and by knife cuts. Successive stages in the development of all these overgrowths are described and illustrated.

The following records were kept: Air temperature and humidity, soil temperature and moisture, rainfall, development in height and diameter of both first- and second-season nursery apple trees, incubation periods of both the crown-gall and the hairy-root bacteria, occurrence of hairy root following inoculation and under natural conditions, and groups of insects prevalent in the soil. During this study different seasons have been relatively wet, dry, or intermediate.

The incubation periods for crown gall and hairy root have been relatively long in the spring and fall and relatively short in the summer. They were apparently correlated with temperature and with active growth of the trees.

Both crown gall and hairy root developed after suitable incubation periods following inoculations made at grafting time. Adhesive tape wrappers delayed the appearance of symptoms slightly but did not reduce the number of infections which appeared.

Mixtures of various overgrowths appeared, especially on the second-year trees.

The evidence available points to soil insects, including white grubs, wireworms, and fungus gnats, as important agents in opening infection courts for bacteria during the growing season.

Although a small amount of the natural occurrence of hairy root was traced to infection at the time of grafting and was doubtless important as a source of inoculum, most of it seemed to follow natural infection during the middle and latter part of the first growing season and during the second growing season. Much of the natural infection appeared to be correlated with warm weather, active growth of the nursery trees, short incubation periods, decreased protection of the unions by wrappers, and insect activity.

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HAIRY ROOT, CROWN GALL, AND OTHER MALFORMATIONS AT THE UNIONS OF PIECE-ROOT-GRAFTED APPLE TREES AND THEIR CONTROL[1]

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INTRODUCTION

The control of hairy root (*Phytomonas rhizogenes* Riker et al.), crown gall (*P. tumefaciens* (Smith and Town.) Bergey et al.), wound overgrowth, and perhaps other enlargements that occur at the unions between scion and root on nursery apple trees grown from piece-root grafts has been attempted in a number of different ways. These attempts have been stimulated by the fact that very commonly the susceptible varieties of nursery trees have shown these enlargements to a serious extent at digging time. In some extreme cases practically a whole planting has been lost. The prevalence of these diseases has been largely responsible for a change in the method of propagation followed by many of the nurserymen east of the Mississippi River. In this region apple trees are propagated commonly by budding, a method which largely obviates the problem of enlargements at the union and which may give a better stand. However, this method of propagation is reported to be more expensive, to require labor during a very busy part of the growing season, and to provide nursery trees on seedling roots. These factors have caused many of the apple-tree growers in the Middle West to continue piece-root grafting despite the loss from these diseases. To find a means of reducing the loss has been the object of the present studies. Several preliminary accounts on certain phases of this work have already appeared (*23*, *25*, *28*, *32*, *33*).[2]

ECONOMIC IMPORTANCE

The actual harm done to the nursery apple trees by enlargements at the union has been discussed by several writers, including Stewart (*45*) and Jakovlev (*13*). However, much of the work reported on the injury caused by graft knots[3] is open to question because of the inadequate diagnosis of the malformations studied. As discussed later, the several different kinds of graft knots that occur doubtless produce various effects upon the trees. More evidence is needed on this question of injury. Since the differentiation of certain kinds of graft knots has been accomplished (*18*, *19*, *26*, *27*) the influence of hairy root on the growth of the trees has received some attention. Comparatively little evidence, beyond that already noted by Riker

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[2] Reference is made by number (italic) to Literature Cited, p. 937.

[3] A general term for any overgrowths at the union.

and Keitt (*31*), has been secured as yet by the present writers on the influence of crown gall and wound overgrowth of known identity on the growth of nursery trees.

Some data on the effect of hairy-root infection on the growth of nursery apple trees were accumulated in 1929 and 1930 (*27*). Second-season trees of the Yellow Transparent variety were employed. Smooth trees and corresponding hairy-root trees that had become naturally infected the previous season were chosen for measurements. On May 17, 1929, the average height and diameter of 100 smooth trees were, respectively, 32.1 and 0.37 inches. Measurements were made of the same trees September 17, 1929. A few of the healthy trees had become infected during the season and were omitted from the final results. The average height and diameter of the smooth trees were, respectively, 51.8 and 0.59 inches; those of the hairy-root trees were 49.2 and 0.56 inches. These averages show a slight advantage in growth during the season, of 2.6 inches in height and 0.03 of an inch in diameter, in favor of the smooth trees. Repetitions of these trials during the season of 1930 also showed a slight advantage, 5.1 inches in height and 0.08 of an inch in diameter, in favor of the smooth trees. These results are in general accord with those of various writers, including Fracker (*6*), Swingle and Morris (*47*), and Muncie (*18*); the last-named reported a reduction in water-flow through woody knots. However, the data presented in the present paper are so limited and the variations among corresponding trees so great that the differences found seem to have little if any significance. These data, however, raise the question whether the hairy root may perhaps be slightly detrimental to the tree.

In an effort to discover whether the hairy roots were able to function as ordinary roots, a rather severe test was made. On May 17, 1929, 100 hairy-root trees that had become infected following inoculation the previous season were treated. The soil was removed so as to expose the main stem of the tree with as little disturbance as possible to the hairy-root development. The stem was then cut off from the main root system just below the hairy-root development, and the soil was replaced. Ninety-five of the trees thus treated lived throughout the season. By September 17, 1929, they had increased, on an average, 4.5 inches in height and 0.05 of an inch in diameter. Similar trials with like numbers of artificially infected trees in 1930 showed almost identical results, only 5 percent of the trees dying. From May 20 to September 20, 1930, the trees increased, on an average, 6.6 inches in height and 0.12 of an inch in diameter. Smooth trees received similar cuts in corresponding positions. This left them with no roots at all, and of course they died promptly. The fact that the trees supported only by hairy-root developments were able to live shows that these roots have certain functional capacities.

The experiments described in the two preceding paragraphs were made on trees that were grown under comparable conditions in adjacent rows in the nursery. It appears, therefore, that trees which were deprived of all roots except the hairy roots made much less growth than untreated smooth trees or trees that had both natural and hairy roots.

In limited trials following pure-culture inoculations Riker and Keitt (*31*) have found crown gall distinctly detrimental to small nursery apple trees.

Whatever may be the actual influence on the apple trees of the various enlargements at the union, it does not lessen the scope of the problem of control. The problem of these enlargements of trees grown from piece-root apple grafts becomes one of prevention.

These malformations vary considerably not only in their external characters but also in their internal structure.

CAUSES AND DESCRIPTIONS OF OVERGROWTHS

Several different kinds of overgrowths occur on nursery apple trees grown from piece-root grafts. Perhaps the most important are: (1) Infectious hairy root, caused by *Phytomonas rhizogenes* (*27*); (2) crown gall caused by *P. tumefaciens* (*10, 43*); and (3) callus or wound overgrowth, which is nonparasitic (*18, 19, 30, 31*). An understanding of the nature of these formations is obviously desirable in making a satisfactory approach to the problem of their control.

Hairy root is the most common enlargement found in some nurseries. It consists usually of a mass of fleshy or fibrous roots that spring from more or less conspicuous enlargements on the main stem. These enlargements seem to have their origin at the union or at some other point of injury through which the bacteria may have gained entrance. The enlargement at the base of the root formation is usually quite hard, owing to irregular masses of woody elements which seem to be directly connected with the vascular system of the main stem. Hairy-root enlargements have been described by various writers, including Hedgcock (*10*), who called them woolly knots to distinguish them from other overgrowths, and recently they have been described in relation to *Phytomonas rhizogenes* by Riker et al. (*27*). These parasitic hairy-root developments should not be confused with other hairy-root formations which apparently are nonparasitic (*18, 24, 31*).

Crown gall occurs as a result of infection by *Phytomonas tumefaciens*. The causal bacteria may supposedly gain entrance to the tissue at the time the graft is made, or through wounds produced at some later period (*18, 31, 38, 41*). The galls proper on apple are comparatively smooth and as a rule do not have roots growing directly from the surface, although roots frequently grow from the main stem around the edge of the gall. Ordinarily the surface of these galls is not covered with a true bark but either with a layer of dead cells or with actively growing gall tissue. The interior is usually soft like cortical tissue but may contain hard woody elements. Galls of this description render infected trees unsalable, but the small percentage of such galls on nursery apple trees in the Middle West makes them of little economic importance. A more complete description of these crown galls has been given by several writers, including Smith et al. (*43*), Riker and Keitt (*31*), and Muncie (*18*).

Callus or wound-overgrowth development begins commonly on the cut surfaces of the scion and root after they have been fitted together and stored for some time in moist packing material. This formation of callus is of course necessary to establish a union between the scion and the root. However, a union may be imperfect because of a variety of influences, such as a poor fit in grafting, a scion larger than the root, loose wrapping, irregular callus development, and the formation of cork over the callus tissue (*10, 18, 31, 35*). In many

cases the development of callus may continue over a long period and may result in an enlargement of sufficient size to deform the tree. Enlargements of this character have been stimulated by girdling young trees with wire or with a wrapper that failed to rot in a comparatively short time (*24*, *29*, *30*). They are caused apparently by the blocking of the downward flow of elaborated food by the girdle and the activity of the tissue immediately above the point of restriction. Apparently a similar condition occurs where the union between the scion and the root is relatively imperfect. As explained later, it has appeared from the cause of these malformations that they might be controlled, as pointed out by Hedgcock (*10*), if a better union between scion and root could be secured and the formation of excess callus prevented by carefully fitting the grafted parts and by using suitable wrapping.

Mixtures of these different types of development occur with more or less frequency from time to time. Complete intergradations between them may be found in many nurseries. Likewise, inoculations with mixtures of the crown-gall and hairy-root organisms have given complete intergradations of the crown-gall and hairy-root symptoms (*24*). Perhaps the most common mixture encountered in the average nursery is that of hairy-root and wound overgrowth. These occur either from the infection of wound overgrowths with hairy root or from the formation of overgrowths at hairy-root infections, doubtless in large part because of the same factors that induce overgrowths above mechanical injuries or girdles. The relative frequency of different types of mixtures has been found to vary in different localities.

Although hairy root, crown gall, and wound overgrowths are apparently the more important types of graft knots, other kinds of overgrowths may occur with considerable frequency under certain conditions. Among these may be mentioned (1) burrknots, described by a number of writers, including Birmingham (*1*), Brown (*2*), Swingle (*46*), Hatton et al. (*9*), Carne (*3*), and Siegler and Piper (*40*), the last-named workers producing malformations morphologically identical with their apple strain of bacteria but failing to isolate these bacteria from naturally occurring burrknots; (2) incompatible unions, mentioned by Riker (*22*); and (3) noninfectious hairy root described by Muncie (*18*), Riker et al. (*27*), and others, the cause of which still remains obscure.

Since the relative prevalence of the different kinds of graft knots has a definite bearing upon the types of control measures that should be employed, their distribution in the nursery was examined.

DISTRIBUTION OF OVERGROWTHS IN THE NURSERY

Malformations, particularly at the unions, on trees grown from piece-root apple grafts appear to occur wherever apple trees are propagated by this method. There has been some confusion about the relative distribution of these malformations because of the difficulty of diagnosis. It is only within the last few years that the causal differences in hairy root, crown gall, and callus developments have been noted. In the survey reported by Riker and Keitt (*31*) crown gall was separated from wound overgrowth and hairy root. However, for the most part these writers placed both wound over-

growths and hairy root in the same classification without distinction. Muncie and Suit (*19*) have reported the results of extensive surveys in which they found that infectious hairy root was of comparatively small consequence. In the light of other investigations (*25, 27, 37*) where the cause and symptoms of infectious hairy root are more clearly defined, it appears that this disease is of great economic importance, especially under some conditions.

The distribution in the nursery of the various malformations under consideration on the underground parts of nursery apple trees has been studied by the present writers in several different localities. Each tree in the nursery row was examined after being dug, and the relative positions of the smooth and the knotted trees were noted. If an overgrowth was found, its size, position, and character were recorded in detail. Two examples of very common types of occurrence and distribution are given in figures 1 and 2. The actual field data have been so arranged that much about the condition of each tree might be indicated by two letters. A comparatively light incidence of overgrowths often shows the type of distribution illustrated in figure 1. This record was taken in one long continuous row of which the trees recorded comprise only a small part. A common distribution showing a heavy incidence of overgrowths appears in figure 2. Results similar to those just cited (figs. 1 and 2) were secured in most of the studies on control reported later (tables 1 to 5). The details of these records are omitted because of their large volume.

These records, of which figures 1 and 2 are small and condensed examples, suggest that (1) if the hairy-root organism comes from the soil where the grafts are planted it is well distributed there; (2) if it comes in with the grafts it is also well distributed among the grafts; and (3) after the disease develops it may spread somewhat along the nursery row. The question of the source of hairy-root inoculum has received detailed consideration in other papers (*11, 29*). Crown gall was found only occasionally. In most of the studies reported in this paper it comprised less than 1 percent of the overgrowths. Excess callus or wound overgrowths occurred with relative frequency on the first-year trees; on second-year trees they were often either healed over or infected with hairy-root bacteria; on still older trees they frequently occurred in combination with hairy root.

DISTRIBUTION OF OVERGROWTHS ON TREES

Most of the overgrowths on the underground parts of the main stems of the trees occur at the union between scion and root, as shown in figures 1 and 2. Further records of 54 trials on trees grown from grafts wrapped with string and of 54 corresponding trials on trees wrapped with adhesive tape are shown in table 1. These trials were made in Iowa, Kansas, Minnesota, Missouri, Nebraska, Oklahoma, and Wisconsin on 1- to 4-year-old trees of the following varieties: Bayfield, Black Ben, Delicious, Dudley, Early Harvest, Fameuse, Florence (crab), Gano, Golden Winesap, Goodhue, Jonathan, McIntosh, Northwestern Greening, Oldenburg, Perkins, Red Siberian (crab), Red Wing, Wealthy, Whitney (crab), Yellow Transparent, and York Imperial.

On an average, less than 5 percent of the trees had overgrowths on the scions and less than 2 percent on the roots, whereas approximately 25 percent of the trees grown from commercial string-wrapped grafts, and from 7 to 14 percent of the tape-wrapped trees, had graft knots at the unions. There was considerable variation in individual trials.

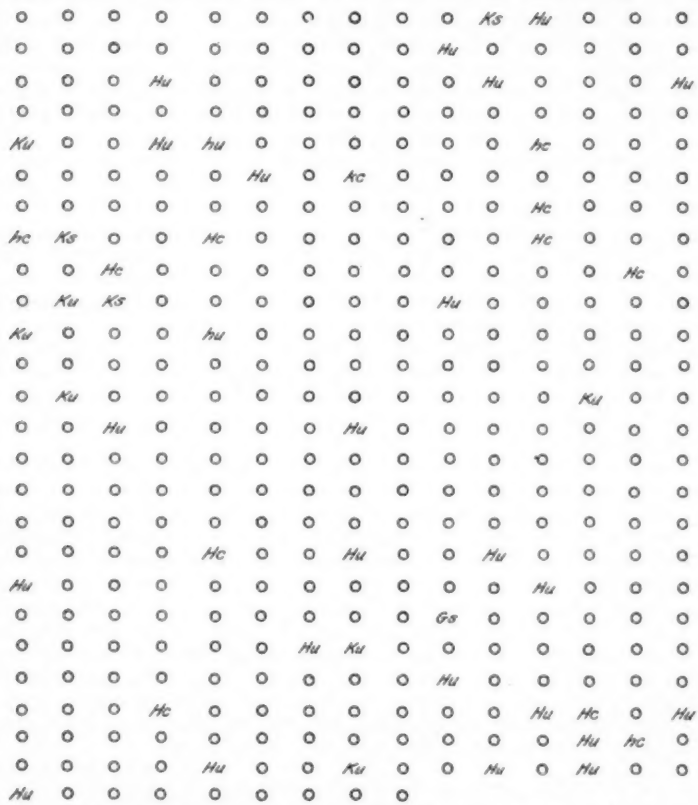

FIGURE 1.—Distribution of enlargements in one continuous row (reading from left to right) on 2-year-old Yellow Transparent apple trees propagated in Iowa from tongue grafts. The characters of the individual trees are summarized according to the following symbols: *, Smooth; *G*, large crown gall; *H*, large hairy root; *h*, small hairy root; *K*, large callus or wound overgrowth; *k*, small callus or wound overgrowth; *c*, located on the scion; *u*, located on the union; *s*, located on the stock.

From 0 to 18 percent of the trees had overgrowths on scions, from 0 to 93 percent on unions, and from 0 to 7 percent on roots. These results confirm those of a number of earlier papers. However, the percentages of overgrowths at the union in these trials were lower than those reported by several workers, including Doidge (*4*) and Muncie (*18*). This difference is probably due to recent improvements in grafting technic and in cultural practices.

Some difficulty was experienced in differentiating incipient hairy root from burrknots on the scions of such varieties as Bayfield and

Okabena, on which burrknots occur frequently. Such burrknots have not been included in these records. Overgrowths were found from time to time on the lateral roots. In some cases they appeared to follow mechancial injury incident to cultivation, but more frequently they were in positions where only insects could have reached them. This subject is discussed more fully by Hildebrand (*11*).

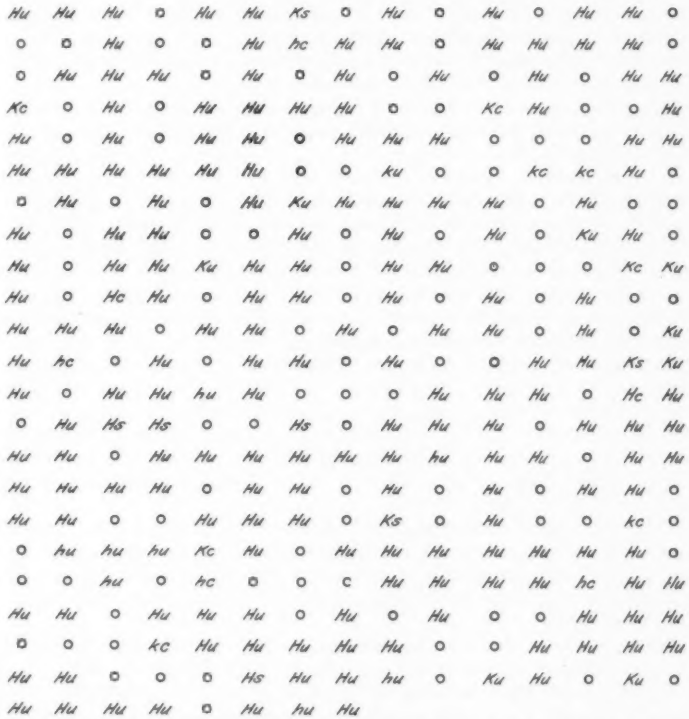

FIGURE 2.—Distribution of enlargements in one continuous row (reading from left to right) on 2-year-old Wealthy apple trees propagated in Kansas from tongue grafts. The characters of the individual trees are summarized according to the symbols shown for figure 1.

TABLE 1.—*Relative frequency of occurrence of enlargements on various underground parts of nursery apple trees grown from piece-root grafts, 1929–31*

Year	Trials	Wrapper	Trees examined	Trees with enlargements on—		
				Scion	Union	Stock
	Number		*Number*	*Percent*	*Percent*	*Percent*
1929	18	String	8,469	4.6	24.8	0.8
	18	Tape	11,848	3.0	7.2	1.1
1930	24	String	8,504	1.1	25.1	1.0
	24	Tape	8,760	1.1	14.2	1.8
1931	12	String	4,132	3.2	25.0	.5
	12	Tape	4,984	3.1	13.6	1.3

CONTROL OF OVERGROWTHS

METHODS AND MATERIALS

In experiments for the control of the various overgrowths on the unions of piece-root-grafted nursery apple trees, four factors have been considered: (1) The kind of seedling employed, (2) the treatment of seedling roots with various antiseptics, (3) the manner of making the graft, and (4) the material used for wrapping the graft. As is pointed out later in this paper and in more detail by Hildebrand (*11*) and Riker and Hildebrand (*29*), wounds produced by soil insects represent another factor deserving special consideration.

The practice of excision of overgrowths and subsequent antiseptic treatment is not considered in the present paper, because of previous unpromising results.

A considerable range of conditions was sought in making the different trials. For this reason tests were conducted in representative nurseries in a number of States, namely, Iowa, Kansas, Minnesota, Missouri, Nebraska, Oklahoma, and Wisconsin. The complexity of the problem and the multiplicity of factors involved have hindered the rapid progress of the work and must be considered in the interpretation of the results secured. Various factors have operated alone or in combination in different nurseries during the same season and in the same nursery in different seasons. Certain control measures successful in one place have failed completely in another. Consequently, corresponding variability has been introduced into repetitions of the trials, the more important trials having been repeated in several places in the same season and in the same place in different seasons. The trial grafts differed from the corresponding control grafts in only one particular.

The varieties of trees employed differed in different localities. In general, varieties grown in comparatively large numbers and those reported to be susceptible to enlargements at the union were chosen for the tests, but some of the less popular and less susceptible varieties were used also.

The age of the trees used differed in different localities. In the Northern States the trees stood 3 or 4 years in the nursery; in the Southern States they were lifted after 1 or 2 growing seasons. In a number of cases trees grown only 1 season were examined in order to secure a preliminary idea of the efficiency of the methods being employed. The examination was made by removing the soil from around the tree until the union could be seen. When this was done, special care was taken not to injure the trees, and after the examination the soil was replaced. Because of the labor involved, especially during wet and cold weather, only a small number of trees were examined, usually 100 receiving the same treatment and 100 controls; in some cases only 50 of each. These examinations were made at random in the larger plantings.

Most of the records of the different trials were kept in considerable detail. The presence or absence of an overgrowth on the underground parts of each tree was noted. Records were kept of the location, relative size, probable cause, and character of tissue of all the enlargements and of the presence, number, character, and size of hairy roots. However, in a number of cases only a summary of the condition at the union was recorded, especially when a representative

distribution had been secured for a variety in a given locality and when the numbers involved were large.

Because of the great volume of the records only synopses of the data are represented here, except in one representative instance.

RELATION OF TREES TO CONTROL FACTORS

The age of the nursery apple trees has an important bearing upon the significance of the results secured. In accord with the results of Melhus and Maney (*16*), the percentage of graft knots on 1-year-old trees was found to be of doubtful value in predicting the percentage on the same trees in later years, but is important in showing the efficiency of control measures applied at grafting time. A comparison of the results with 1-year-old trees and the same trees when 2 years old shows that important factors continue to operate in the second season.

Comparisons between the percentages of smooth unions on first- and second-season trees, some of which had been wrapped with tape and others with string, are shown in table 2. The 10 trials involved records of data on 14,435 trees. The observations on first-season trees were made by direct examination after the soil had been removed from the unions. The soil was then replaced. Because of the labor involved, a comparatively small number of trees in each lot (between 50 and 100 taken at random through the entire planting) were examined.

TABLE 2.—*Percentages of smooth unions on grafts of various plantings of nursery apple trees at different ages*

State	Variety	Amount of graft knot	Smooth unions on trees wrapped with—							
			Tape				String			
			First season	Second season	Third season	Fourth season	First season	Second season	Third season	Fourth season
			Pct.	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*
Kansas	Wealthy	Great	96	77	.....	.....	54	37	.....	.....
	do	do	86	67	.....	.....	34	39	.....	.....
Minnesota	do	do	100	94	.....	.....	46	48	.....	.....
	Okabena	do	100	58	.....	.....	16	4	.....	.....
Iowa	do	Small	95	99	.....	.....	55	74	.....	.....
	Oldenburg	do	100	98	.....	.....	68	74	.....	.....
	do	do	92	94	.....	.....	56	79	.....	.....
	Yellow transparent	do	100	97	.....	.....	84	89	.....	.....
	do	do	91	96	.....	.....	82	88	.....	.....
	Wealthy	do	97	90	.....	.....	84	86	.....	.....
Nebraska	do	do	96	.....	81	.....	70	.....	81	.....
	do	do	100	.....	94	.....	92	.....	82	.....
Minnesota	Bayfield	Great	84	.....	.....	49	48	.....	.....	18
	do	do	82	.....	.....	36	58	.....	.....	29
	Wealthy	do	92	.....	.....	59	40	.....	.....	14
	do	do	100	.....	.....	67	46	.....	.....	34
	Okabena	do	84	.....	.....	44	36	.....	.....	13
	do	do	100	.....	.....	42	16	.....	.....	8

When the amount of graft knot was large, several results were noted which deserve comment. Infectious hairy root was the predominant type of overgrowth. The trees wrapped with tape generally appeared to be protected during the first year against the several factors causing the development of graft knots, but this protection had

little influence during the second year when the agencies producing graft knot continued to operate. The question as to just what these agencies were and the method by which they acted has been discussed in more detail by Riker and Hildebrand (*29*). These writers have also reported that the possibility of long incubation periods for bacteria on actively growing trees under favorable conditions seems too remote to have much bearing upon this question. Siegler and Piper (*40*) have reported that causal bacteria could be isolated rarely if at all from aerial crown-gall inoculations after 65 days.

When the amount of graft knot was relatively small it appeared that many of the unions wrapped with string which had shown wound overgrowths or excess callus by the end of the first year became smooth by the end of the second year. This process had been noticed earlier by Melhus and Maney (*16*), with respect to poor unions. The time required for excess callus to become smooth depended upon the size of the callus, the vigor of the tree, and the absence of other knot-producing agencies. No cases were noted of recovery from infectious types of overgrowths.

Comparisons between the percentages of smooth unions on trees of different ages, e.g., on first- and third-season trees and on first- and fourth-season trees, gave similar results. A total of 8,906 trees was examined in these trials.

These results have a definite bearing upon the interpretation of the various control studies. It appeared quite clear that the first season is not necessarily the most important for the development of graft knots; however, it may be the most important for interpreting the results of the control measures applied at grafting time. When the amount of graft knot was large and the graft knots were predominantly of the infectious hairy-root type, the first season was often less important than the seasons that followed. On the other hand, when the amount of graft knot was small and the graft knots were mostly of the wound-overgrowth type, there was often no material change from the first season. Except during an epidemic of hairy root, the percentages of smooth unions following string wrapping increased during the second season; however, they never reached the point where they exceeded the percentages of smooth unions following tape wrapping. No epidemics of crown gall were observed. Since control measures thus far have been applied almost entirely at grafting time and during the first season, these studies show that under certain conditions the methods used cannot be expected to yield fully satisfactory results. However, they can, perhaps, reduce the intensity of an epidemic by lowering the percentages of trees that provide inoculum for later years. Thus far there is no evidence to show that the crown-gall (*31*) and hairy-root (*11*) bacteria may gain entrance into the host tissue in any way except through wounds. Consequently, these studies point to the root-chewing insects in the soil and to injuries during cultivation as agents in the production of epidemics, certainly after the first season and probably during the first season.

INFLUENCE OF SEEDLING STOCK USED

To determine what influence the seedling stock might exert on the number of enlargements that develop from piece-root grafts, 32 trials were made involving 31,000 grafts. Seedlings from France and from

the States of Colorado, Kansas, Vermont, and Washington were compared. The trials were made during several years, in Iowa, Kansas, Missouri, Nebraska, and Oklahoma, with scions of the following varieties: Rome Beauty (dark red sport), Delicious, Golden Delicious, McIntosh, and Wealthy. The details of these studies are omitted because of their volume.

In all but nine of the individual trials the percentage differences of smooth trees on grafts with different seedlings were relatively small. When the percentage differences were 10 or more the values are listed as examples to show the variations. In these trials the percentage differences in smooth trees obtained from the use of different lots of seedling stock were 18, 32, 12, 35, 32, 12, 10, 11, and 11. The first five of these examples showed a predominance of infectious hairy root. In these cases it seems clear that in each trial one lot of the seedlings employed carried the hairy-root organism on the surface to a much greater extent than the other. Out of the 64 lots of seedlings employed in the 32 trials, 39 lots yielded over 80 percent of smooth trees when they were lifted. The relatively large percentage of smooth trees and the diversity in type of the enlargements that were found suggest that hairy-root bacteria on the surface of seedling roots was not a very serious factor in a majority of the cases studied. Although different lots of seedlings showed considerable variability, no correlation in the amount of graft knot was found between one year and the next with the seedlings from one region or even from one nursery. But in several cases it was found that various shipments of seedlings from a particular nursery gave consistently unfavorable results in several different States in the same year. However, the next year the seedlings from that same nursery were among the best employed. To summarize these results, it appears that, in accord with the results of Waite and Siegler (*49*) and Siegler and Piper (*41*), circumstantial evidence was found indicating that the causal bacteria may be carried on the surface of certain lots of apple seedlings. It therefore seemed desirable to try isolations from the surfaces of suspected seedlings.

Out of 19 attempts at isolation, positive results were secured in 15; repetitions of these trials gave similar results. The hairy-root bacteria were obtained from seedling apple roots in 24 out of 26 trials. The identity of the bacteria was determined by certain cultural characters and by successful inoculations into plants.

Other factors besides the presence of hairy-root bacteria should be mentioned in considering the incidence of overgrowths in grafts made with different lots of seedlings. The physiology of the seedling in relation to ripeness and to resistance is well worthy of further study. Attention has been given by several writers (*8, 11, 34, 42*) to the question of resistance of different varieties of fruit trees to crown gall and related diseases. It therefore seemed desirable to determine whether apple seed from different sources would produce seedlings that differed in resistance to graft knots.

The exact source of the seed was not easy to determine. However, in a few instances the seed was followed from the variety of apple that produced it, and in three cases the seed was traced to individual trees. Two experiments with seed from Hopa (crab) and Meader trees may be mentioned together. The special seed was planted in the same field with commercial seed, and the seedlings were used to make piece-

root grafts with McIntosh scions. At the end of two growing seasons from the time the grafts were planted, data were recorded for 500 trees from each seed source. The trees grafted on seedlings grown from Hopa seed were 89 percent smooth; those from Meader seed also were 89 percent smooth. Those from four corresponding lots of commercial seed were, respectively, 58, 56, 56, and 61 percent smooth. In a similar trial involving similar numbers, seed from Wealthy trees was employed to grow seedlings on which were grafted Jonathan scions. The trees grafted on seedlings grown from Wealthy seed were 91 percent smooth. Those from three corresponding lots of commercial seed yielded, respectively, 74, 78, and 78 percent smooth trees. Although these results are too few to justify conclusions, they are sufficiently striking to show the desirability of further work in this direction. Such work presents the difficulty, however, of differentiating between the amount of true resistance and the amount of response to causal bacteria carried on the surface or within small injuries. Although these Hopa, Meader, and Wealthy seedlings received the same treatment as the commercial seedlings, the possibility remains that they may merely have escaped surface contamination by the hairy-root bacteria.

VALUE OF CERTAIN ANTISEPTICS

The presence of the hairy-root bacteria on the surfaces of certain lots of seedlings points to the possible value of antiseptic treatments for seedling roots.

Certain antiseptics appear to have promise at times in preventing infection when the grafts are made from seedlings carrying undesirable bacteria. Since Waite's work in 1909,[4] antiseptics have been employed with varying success by a number of workers (*7*, *10*, *12*, *15*, *16*, *20*, *31*, *36*, *44*, *48*, *49*, and *50*). The present writers made trials with a considerable number of antiseptics in the hope of controlling the various overgrowths due to bacteria that gain entrance into the plant at grafting time. However, since it has been determined, as explained earlier, (1) that not all graft knots are due to bacteria, (2) that not all seedlings carry infectious bacteria in significant numbers, and (3) that a considerable percentage of the graft knots are caused by hairy-root bacteria that gain entrance to the tissue months after the union is established (*11*, *29*), it is not surprising that the results of work with antiseptics have failed to be uniformly promising.

A considerable number of substances were given laboratory, greenhouse, and field trials. The best of these were selected for more extensive trials. Thirty-five treatments, with corresponding controls, were made in the preparation of approximately 25,000 grafts in Iowa, Kansas, Minnesota, Missouri, Oklahoma, and Wisconsin with scions of the following varieties: Rome Beauty (dark red sport), Jonathan, Stayman Winesap, Wealthy, and Yellow Transparent. Since most of the antiseptics were not found to be satisfactory, mention is made of only three.

Hydroxymercurichlorophenol sulphate (Semesan) was used in 15 trials, which are summarized in table 3.

[4] It appears that Waite was the first to employ an antiseptic (formaldehyde) solution as a dip for apple stocks and scions before grafting. Although this early work was unpublished it has already been noted (*49*).

TABLE 3.—*Effect of three antiseptics on occurrence of malformations at the unions of piece-root-grafted nursery apple trees*

Antiseptic	Trials	Age of trees	Wrapper	Total trees examined	Stand	Trees showing indicated condition at union		
						Smooth	Small knot [a]	Large knot [b]
	Number	*Years*		*Number*	*Percent*	*Percent*	*Percent*	*Percent*
Semesan	10	1–3	String	2,172	52	74	8	18
Controls	10	1–3	do	2,666	52	70	10	20
Semesan	5	1–2	Tape	1,675	66	85	1	14
Controls	5	1–2	do	2,550	63	81	1	18
Silver nitrate	6	1–3	String	2,150	67	86	4	10
Controls	6	1–3	do	2,676	53	82	6	12
Silver nitrate	8	1–3	Tape	4,098	54	95	2	3
Controls	8	1–3	do	4,204	66	94	1	5
Mercuric chloride	2	2	do	834	64	94	1	5
Controls	2	2	do	1,792	64	80	0	20

[a] This class included all enlargements, regardless of cause, smaller in cross measurements than half the diameter of the tree.

[b] This class included all enlargements larger in cross measurements than half the diameter of the tree.

In 10 of the trials the grafts were wrapped with string or cloth and treated in a 1:400 solution according to the directions of Waite and Siegler (*49*). In the 5 other trials only the seedlings were treated and the grafts were wrapped with adhesive tape. The control grafts received no treatment whatever. In one trial in Minnesota, after 1 season of growth with a string wrapper, the treated grafts showed 68 percent smooth, whereas the untreated grafts showed 42 percent smooth. In another trial, in Missouri, after 2 seasons of growth with a cloth wrapper, the treated grafts showed 56 percent smooth and the untreated grafts 88 percent smooth. The Minnesota trial showed a difference of 26 percent in favor of the treatment; whereas the Missouri trial showed a difference of 32 percent against the treatment—great and contradictory differences. However, in most cases the differences were rather small and more of them were in favor of the treatment than against it. The averages given in table 3 show several percent in favor of the treatment. The average differences are considered to be within the range of experimental error. The stand appeared not to be influenced by the treatment. These results appear in general accord with the following statement by Siegler (*39*): "We experimented with the use of Semesan which gave good results in earlier years but which recently has not proved so efficacious." As explained earlier, the factors that operate to induce overgrowths in one place may be very different from those in another. The case in which 32 percent more enlargements were induced in the treated grafts than in the controls was very puzzling. An explanation was sought in a laboratory study of this substance.

The efficiency of the hydroxymercurichlorophenol sulphate was determined against the crown-gall organism with a modified Rideal-Walker technic as used by Keitt, Shaw, and Riker (*14*). In the absence of any organic matter a dilution of 1 in 800 at 20° C. killed a measured concentration of a 48-hour-old washed culture of the bacteria in 10 minutes, but not in 5 minutes. As was to be expected, the presence of a little soil extract or other organic material considerably reduced the efficiency of this antiseptic. It seems likely that, under nursery conditions, enough soil might easily be present to counteract

the germicidal effect of the chemical. Under such conditions it appears probable that the treatment might spread rather than destroy any bacteria present.

Silver nitrate, 1:1,000, gave rather poor results in the control of overgrowths. The seedling roots were dipped in this substance and held for 2 minutes; no subsequent treatment was made. In 6 trials the grafts were wrapped with string and in 8 with tape. A summary of these trials is included in table 3. The differences between the percentages of smooth trees resulting from the treated grafts and those resulting from the untreated grafts somewhat favor the treatment but are still within experimental error. However, the injury from the silver nitrate produced a noticeable reduction in stand.

The statistical method of Fisher (*5*) was applied to these data, in consultation with Dr. R. A. Brink, of the University of Wisconsin, in order to see whether the treatments might be more effective than they appeared. The significance of the mean difference in relation to the standard deviation of the difference was calculated. For treatments with hydroxymercurichlorophenol sulphate and with silver nitrate, the values of P were 0.7 and 0.6, respectively. These calculations, which are omitted because of their large volume, corroborate the conclusion derived from inspection that these treatments had little if any effectiveness in general practice.

Mercuric chloride, 1:1,000, in limited trials has given more promising results than any other antiseptic employed. The average of two trials shown in table 3 indicates that if used in conjunction with tape wrappers it may be effective, especially if the seedlings carry infectious bacteria. Other trials now under way appear thus far to corroborate these results.

Tested by the laboratory method described above, mercuric chloride showed considerably greater toxicity than Semesan. A dilution of 1 in 15,000 at 20° C. killed a measured concentration of a 48-hour-old culture of crown-gall bacteria in 10 minutes, but not in 5 minutes. Further work with antiseptics seems desirable.

COMPARISON OF WELL-MATCHED TONGUE AND WEDGE GRAFTS

The type of graft in relation to the prevention of various overgrowths at the union of piece-root apple grafts was examined in a number of trials. Hedgcock (*10*) reported that poorly made grafts were more likely than well-made ones to produce callus enlargements and were also likely to become infected. In addition, Riker and Keitt (*31*) showed that poorly made grafts united only a part of the scion to the root and that a situation similar to a partial girdle resulted. Siegler (*38*) suggested that the fit of the grafts has been overemphasized. Melhus et al. (*17*) found that better unions might be secured with wedge grafts. A number of different series of well-fitted tongue and wedge grafts were made. Various lots of these grafts were wrapped with adhesive tape; others were wrapped with string. The results of these tests are presented in condensed form in table 4.

These studies involved 72 different trials, with a total of 27,981 tongue grafts and 21,759 wedge grafts. The results were obtained in 1928 to 1931, inclusive. The experiments were made in Iowa, Kansas, Minnesota, Missouri, Oklahoma, and Wisconsin on the following

varieties: Bayfield, Rome Beauty (dark red sport), Delicious, Dudley, Early Harvest, Golden Delicious, Jonathan, Maiden Blush, Okabena, Oldenburg, Red Wine, Wealthy, Whitney (crab), and Yellow Transparent. The examination of first-year trees, as already explained, was made by removing the soil from around the unions of trees taken at random and not by lifting the trees. The data on comparative lots of 1-year-old trees were taken under one set of conditions, while those on 2-, 3-, and 4-year-old trees were taken under different conditions. Consequently, comparisons are possible between trees of the same age, but not between trees of different ages.

TABLE 4.—*Effect of tongue and wedge grafts on occurrence of malformations at the unions of piece-root-grafted nursery apple trees*

Wrapper	Trials (Number)	Graft	Age of trees	Total trees examined	Stand	Trees showing indicated condition at the union		
						Smooth	Small knot [a]	Large knot [b]
			Years	*Number*	*Percent*	*Percent*	*Percent*	*Percent*
Tape	14	Tongue	1	1,893	69	96	3	1
	14	Wedge	1	1,485	58	93	4	3
	13	Tongue	2	7,716	53	92	1	7
	13	Wedge	2	6,265	65	92	1	7
	7	Tongue	3	3,505	71	89	0	11
	7	Wedge	3	3,072	55	89	0	11
	3	Tongue	4	630	.....	48	4	48
	3	Wedge	4	464	.....	51	5	44
	Summary:							
	37	Tongue	1–4	13,744	65	89	2	9
	37	Wedge	1–4	11,286	60	89	2	9
String	10	Tongue	1	926	65	59	17	24
	10	Wedge	1	850	57	62	18	20
	14	Tongue	2	8,459	75	74	4	22
	14	Wedge	2	6,681	63	75	3	22
	8	Tongue	3	4,249	71	73	1	26
	8	Wedge	3	2,449	69	72	1	27
	3	Tongue	4	603	.....	24	4	72
	3	Wedge	4	493	.....	15	5	80
	Summary:							
	35	Tongue	1–4	14,237	70	65	7	28
	35	Wedge	1–4	10,473	61	65	7	28
Tape and string	Summary:							
Do	72	Tongue	1–4	27,981	68	78	4	18
	72	Wedge	1–4	21,759	60	78	4	18

[a] This class included all enlargements, regardless of cause, smaller in cross measurements than half the diameter of the tree.
[b] This class included all enlargements larger in cross measurements than half the diameter of the tree.

Considerable variations in the percentage of overgrowths at the unions were found in both directions in the comparison of tongue and wedge grafts. These variations occurred whether the grafts were wrapped with string or tape. It appeared from these trials that there was no significant difference between tongue and wedge grafts in the reduction of overgrowths at the union. These results are not necessarily opposed to those of Melhus et al. (*17*), for apparently these writers were concerned primarily with callus developments, whereas the present writers considered all the various kinds of overgrowths discussed earlier.

The reduced average stand of the wedge grafts wrapped with string as compared with that of tongue grafts seemed to have little signifi-

cance because of the great variations in the results and because these variations favored first one and then the other type of graft. Fisher's method (*5*) was applied to the data on stands of tongue and wedge grafts at the end of the first season, to determine the significance of the mean difference in relation to the standard deviation of the difference. The value of P was 0.4, indicating that the difference had little if any significance. However, it should be noted that wedge grafts wrapped with string were more likely than tongue grafts to come apart before they were planted. Likewise, the wedge grafts had a greater tendency to send up sprouts from the seedling roots. When the grafts were wrapped with tape, the possibility of their coming apart and of the roots sending up sprouts was reduced.

EFFECT OF KIND OF WRAPPING USED

For reducing the percentage of enlargements at the unions of piece-root-grafted nursery apple trees, the kind of wrapping used was the most important single factor studied. A considerable number of different kinds of wrapping were tried, including string, waxed string, chemically treated string, string covered with wax after wrapping, paper, raffia, chemically treated raffia, raffia covered with wax, cloth, waxed cloth, and a number of kinds of adhesive tape. Hedgcock (*10*) found that, of the wrappers he tested, cloth was the best. In the present trials cloth was found to be better than any of the others except adhesive tape. Consequently detailed reports of the long series of trials in which wrappers other than adhesive tape were employed have been omitted. However, certain determinations were made which deserve mention. It was found that by means of various treatments with different chemicals the string wrappers could be preserved for practically any length of time during the growing season. It was also observed that under average conditions a string wrapper that remained longer than 12 weeks was likely to produce girdling. The time necessary for decay of the wrapper varied considerably under different environmental conditions. When the ground was unusually moist the trees grew more rapidly and the wrapper decayed more quickly. Conversely, when the soil was comparatively dry the wrapper lasted longer, but the trees did not grow so rapidly. The strength of the string decreased rather gradually as the season progressed. This diminution apparently depended on the ready access of soil organisms, for any protected portion of the string retained its strength much longer than the unprotected portions.

The adhesive tape chiefly employed was essentially a cloth wrapping to which had been added a plaster mass. The adhesive-tape wrapper provided several valuable features, including (1) reasonable cost, (2) easy application, (3) firm wrapping, which prevented injury to the union during subsequent manipulations and which facilitated handling, (4) a waterproof covering, which prevented the entrance of undesirable material, (5) a mechanical prevention against the development of excess callus on the surface of the union, and (6) a barrier for some months against insect injury at the union.

Not all kinds of adhesive tape were satisfactory. A small number of trials with electrician's friction tape and with tape spread with "surgeon's mass" gave such unpromising results that they were dis-

continued. Certain other special tapes in which various chemicals were incorporated sometimes showed no advantage over ordinary tape. Some of these special tapes had serious disadvantages; a few of them considerably reduced the stands, and two samples that were tried prevented any of the grafts from growing. Several of these special tapes were comparatively slow to decay in the soil and for that reason might last long enough under some conditions to produce girdling. The adhesive tape [5] employed in these trials was rather similar to that used extensively by the medical profession, but with modifications in the interests of economy.

The manner of application of the adhesive tape was found to be important both in relation to speed and to the results secured. Scions 5 to 6 inches long and roots 3 to 4 inches long were used for the grafts, which were well made from good materials. The method of applying the adhesive tape was equally successful with either tongue or wedge grafts. A roll of ½-inch-wide adhesive tape was mounted on a roller at the side of the operator. The graft was turned in the hand and the tape was applied in a spiral wrap over every part of the union and overlapped the edge slightly. Only enough tape was used to make a water-tight covering over every part of the cut surface. The tape was so applied that no more than two thicknesses of material circled the graft at any one point. After a little practice it was possible to wrap 400 to 600 grafts in an hour. The amount of tape used varied with the size of the grafts, but on an average approximately 110 yards of tape one half inch wide wrapped 1,000 grafts.

The tests with wrappers covered a wide range of conditions. Results are reported for the years from 1925 to 1931, inclusive. The unions of the first-year trees taken at random were examined by removing the soil to a suitable depth for inspection. Sometimes only 50 tape-wrapped and 50 string-wrapped trees were examined, but at other times there were 100, 250, or 300 in each lot.

The results from the use of tape wrappers as compared with those from the use of commercial wrappers have been summarized for each trial (table 5) and have been grouped according to the age of the tree at the time the data were taken. As explained earlier, no comparison should be made between the data on trees of different ages, for the trials are not comparable.

Table 5 presents the results of 145 trials with adhesive tape wrappers with a corresponding set of controls. The trials with tape involved 55,326 trees, and the controls involved 55,105. This table shows no significant difference in stand because of the different wrappers. It shows variability in the amount of graft knot according to age of tree, variety, and the State in which the trees were grown. It also indicates a distinct increase in the percentage of smooth trees correlated with the use of adhesive tape. At the same time it emphasizes the variation between individual trials and indicates that different factors operate in the same nursery in different seasons and in different nurseries in the same season.

A summary of table 5 is given in table 6.

[5] Manufactured under the name "nurseryman's tape."

TABLE 5.—*Effect of string* [a] *and adhesive-tape wrappers on occurrence of malformations at the unions of piece-root-grafted apple trees of various ages*

FIRST-YEAR TREES

State	Variety	Unions showing indicated condition on trees wrapped with—							
		String				Tape			
		Total examined	Smooth	Small knot [b]	Large knot [c]	Total examined	Smooth	Small knot [b]	Large knot [c]
		Number	*Percent*	*Percent*	*Percent*	*Number*	*Percent*	*Percent*	*Percent*
Iowa	Oldenburg	50	68	16	16	50	100	0	0
	do	50	56	34	10	50	92	8	0
	Wealthy	100	55	14	31	100	95	3	2
	do	100	84	10	6	100	100	0	0
	do	100	69	18	13	100	99	1	0
	do	100	84	2	14	100	97	2	1
	Yellow Transparent	50	84	8	8	50	100	0	0
	do	50	82	12	6	50	96	2	2
Kansas	Wealthy	50	54	26	20	50	96	2	2
	do	50	34	30	36	50	86	10	4
	do	50	36	16	48	50	86	10	4
	Yellow Transparent	300	71	29	0	300	78	22	0
	do	300	68	32	0	300	71	29	0
Minnesota	Bayfield	50	48	20	32	50	84	6	10
	do	50	58	16	26	50	82	10	8
	Okabena	50	36	26	38	50	84	6	10
	do	50	16	30	54	50	100	0	0
	Wealthy	50	40	20	40	50	90	4	6
	do	50	40	20	40	50	92	4	4
	do	50	46	30	24	50	86	12	2
	do	50	46	30	24	50	100	0	0
	do	50	42	18	40	50	100	0	0
	do	50	42	18	40	50	98	2	0
	do	50	52	14	34	50	94	6	0
	do	100	83	3	14	100	99	0	1
Nebraska	do	100	70	13	17	100	100	0	0
	do	100	70	13	17	100	96	3	1
	do	100	98	1	1	100	98	2	0
	do	100	92	4	4	100	100	0	0
Oklahoma	do	250	7	24	69	250	29	27	44
Wisconsin	do	250	53	27	20	250	94	2	4

SECOND-YEAR TREES

Iowa	Ben Davis	155	72	1	27	215	94	0	6
	Delicious	50	94	2	4	50	100	0	0
	do	50	82	6	12	50	100	0	0
	do	50	84	4	12	50	96	2	2
	do	50	94	4	2	50	96	2	2
	do	50	96	4	0	50	100	0	0
	do	50	86	0	14	50	100	0	0
	do	50	98	0	2	50	96	0	4
	do	50	94	2	4	50	96	0	4
	Oldenburg	992	74	3	23	1,000	98	0	2
	do	399	79	2	19	1,014	94	0	6
	Dudley	50	88	8	4	50	88	0	12
	do	50	94	2	4	50	94	2	4
	Early Harvest	1,005	84	1	15	940	98	0	2
	Fameuse	508	79	2	19	1,009	97	0	3
	Florence (crab)	414	97	0	3	445	99	0	1
	Gano	573	80	0	20	695	97	0	3
	Golden Winesap	768	78	1	21	555	99	0	1
	McIntosh	1,012	84	2	14	1,020	94	1	5
	Red Siberian (crab)	512	96	0	4	575	98	0	2
	Wealthy	935	74	5	21	1,016	99	0	1
	do	50	70	16	14	50	96	2	2
	do	50	78	2	20	50	94	4	2
	do	1,000	93	0	7	1,000	97	0	3
	do	1,000	93	0	7	1,000	98	0	2
	do	1,000	90	0	10	1,000	97	0	3
	do	1,000	90	0	10	500	99	0	1
	do	279	86	0	14	1,330	90	0	10
	Whitney (crab)	1,034	91	0	9	1,049	99	0	1
	Yellow Transparent	259	88	2	10	286	96	1	3
	do	1,180	89	2	9	1,093	97	0	3

[a] Including string, waxed string, and waxed raffia.

[b] This class includes all enlargements, regardless of cause, smaller in cross measurements than half the diameter of the tree.

[c] This class includes all enlargements not classified as small.

TABLE 5.—*Effect of string and adhesive-tape wrappers on occurrence of malformations at the unions of piece-root-grafted apple trees of various ages*—Continued

SECOND-YEAR TREES—Continued

State	Variety	Unions showing indicated condition on trees wrapped with—							
		String				Tape			
		Total examined	Smooth	Small knot	Large knot	Total examined	Smooth	Small knot	Large knot
		Number	*Percent*	*Percent*	*Percent*	*Number*	*Percent*	*Percent*	*Percent*
Kansas	Ben Davis	654	49	4	47	649	60	5	35
	Jonathan	612	74	1	25	579	88	0	12
	do	612	74	1	25	595	91	1	8
	do	588	70	1	29	612	87	1	12
	McIntosh	567	89	1	10	431	95	0	5
	do	559	89	1	10	518	95	0	5
	do	1,005	58	3	39	512	76	2	22
	do	534	55	2	42	515	73	1	26
	do	550	56	4	40	551	80	1	19
	do	112	61	3	36	534	83	0	17
	Wealthy	898	37	7	56	840	77	4	19
	do	771	39	4	57	743	67	4	29
	do	775	35	6	59	743	67	4	29
	do	456	52	3	45	462	71	0	29
	Yellow Transparent	496	45	5	50	502	52	2	46
Minnesota	Okabena	50	4	4	92	50	58	10	32
	Wealthy	50	48	8	44	50	94	0	6
	do	250	62	14	24	250	96	2	2
Missouri	Ben Davis	305	79	1	20	273	92	0	8
	Rome Beauty (dark red sport)	1,000	68	14	18	1,000	89	9	2
	do	1,000	68	14	18	800	78	20	2
	Oldenburg	1,496	85	1	14	731	95	0	5
	Early Harvest	707	93	1	6	137	98	0	2
	Fameuse	1,080	89	1	10	996	97	0	3
	Maiden Blush	648	90	1	9	836	99	0	1
	Wealthy	178	57	2	41	216	80	2	18
	do	243	57	2	41	316	82	3	15
	do	243	57	2	41	342	84	3	13
	do	500	47	1	52	45	87	0	13
	do	500	47	1	52	68	88	2	10
	do	1,000	94	0	6	1,000	98	0	2
	do	1,000	81	1	18	1,000	89	1	10
	do	1,000	46	2	52	1,000	94	1	5
	do	250	74	3	23	250	90	1	9
	Winesap	227	90	1	9	320	97	0	3
	Yellow Transparent	693	47	2	51	583	87	0	13
Nebraska	Wealthy	50	82	6	12	50	92	6	2
	do	50	82	6	12	50	100	0	0
	do	250	66	21	13	250	89	4	7
Oklahoma	Delicious	447	84	0	16	238	98	0	2
	do	438	72	1	27	310	87	2	11
	do	473	94	1	5	465	97	0	3
	Jonathan	436	94	0	6	636	98	0	2
	York Imperial	367	66	1	32	441	89	1	10
Wisconsin	Wealthy	250	72	12	16	250	90	8	2
	do	250	69	22	9	250	95	3	2
	do	250	86	8	6	250	96	2	2

THIRD-YEAR TREES

Iowa	Dudley	174	90	0	10	34	91	0	9
	do	36	94	0	6	92	90	0	10
Minnesota	Wealthy	101	80	0	20	104	94	0	6
	do	270	85	1	14	182	96	0	4
	do	50	64	0	36	50	94	0	6
Nebraska	do	134	66	5	29	120	85	5	10
	do	306	65	11	24	215	91	0	9
	do	600	78	2	20	600	88	1	11
	do	600	78	2	20	600	88	0	12
	do	600	81	0	19	600	93	0	7
	do	600	81	0	19	600	94	0	6
	do	600	80	0	20	600	80	0	20
	do	600	80	0	20	600	89	0	11
	do	376	81	0	19	375	81	0	19
	do	361	82	1	17	382	94	0	6
	Whitney (crab)	533	85	1	14	529	93	0	7
	do	645	86	1	13	742	95	1	4
	Yellow Transparent	961	79	1	20	972	87	1	12
	do	813	84	0	16	765	87	0	13

TABLE 5.—*Effect of string and adhesive-tape wrappers on occurrence of malformations at the unions of piece-root-grafted apple trees of various ages*—Continued

THIRD-YEAR TREES—Continued

State	Variety	Unions showing indicated condition on trees wrapped with—							
		String				Tape			
		Total examined	Smooth	Small knot	Large knot	Total examined	Smooth	Small knot	Large knot
		Number	*Percent*	*Percent*	*Percent*	*Number*	*Percent*	*Percent*	*Percent*
Wisconsin	Bayfield	580	50	3	47	509	67	1	32
	Oldenburg	717	46	1	53	413	65	2	33
	Goodhue	495	60	0	40	542	68	0	32
	Northwestern Greening	402	71	5	24	736	71	2	27
	Perkins	763	66	0	34	310	74	1	25
	Red Wing	634	33	0	67	240	63	0	37
	do	471	66	5	29	482	82	4	14
	Wealthy	300	88	4	8	300	96	2	2
	do	1,243	48	0	52	424	73	0	27

FOURTH-YEAR TREES

Minnesota	Bayfield	161	18	9	73	117	49	6	45
	do	184	29	8	63	265	36	7	57
	Okabena	111	8	1	91	57	42	2	56
	do	60	13	4	83	87	44	3	53
	Wealthy	308	34	5	61	362	67	2	31
	do	308	34	5	61	308	67	4	29
	do	272	14	2	84	331	59	3	38
	do	272	14	2	84	260	59	6	35

TABLE 6.—*Summary of table 5 on experiments showing total or average effect of string*[a] *and adhesive-tape wrappers on occurrence of malformations at the unions of piece-root-grafted nursery apple trees of various ages*

Trials (number)	Wrapper	Age of trees	Total trees examined	Stand	Trees showing indicated condition at the union		
					Smooth	Small knot[b]	Large knot[c]
		Years	*Number*	*Percent*	*Percent*	*Percent*	*Percent*
31	String	1	2,900	57	57	19	24
31	Tape	1	2,900	59	91	6	3
78	String	2	39,555	60	75	3	22
78	Tape	2	38,521	63	91	1	8
28	String	3	13,974	63	73	2	25
28	Tape	3	12,118	59	85	1	14
8	String	4	1,676		21	4	75
8	Tape	4	1,787		53	4	43
Summary:							
145	String	1–4	58,105	59	68	6	26
145	Tape	1–4	55,326	60	88	2	10

[a] Including string, waxed string, and raffia and wax.

[b] This class included all enlargements, regardless of cause, smaller in cross measurements than half the diameter of the tree.

[c] This class included all enlargements larger in cross measurements than half the diameter of the tree.

The superiority of adhesive-tape wrapping for reducing the percentage of enlargements on piece-root-grafted apple trees in most cases is beyond question. In none of the 145 trials in which there was more than 10 percent of disease were the string wrappers found to yield a higher percentage of smooth trees than the tape wrappers.

This alone places the mathematical probability that the tape is valuable in very significant figures.

The degree of superiority of the tape is difficult to estimate because of the variability of the results. In 20 trials there was less than 10 percent of disease development on the checks. Consequently, in such trials, control measures had little chance to show their value. On the other hand, some extreme differences occurred, such as differences of 40, 42, 48, and 56 percent in favor of the adhesive tape. The detailed results show differences ranging from 0 to 58 percent. The significance of the mean difference in relation to the standard deviation of the difference when at least 10 percent of knots were present was calculated, according to the method of Fisher (*5*), for trees of each age. When the value of P was less than 0.05, the mean difference in relation to the standard deviation of the difference was considered significant. The values of P were as follows: For the first-year trees, 0.02; for the second-year trees, 0.09; for the third-year trees, 0.12; and for the fourth-year trees, 0.02. In considering these values the variable factors already mentioned should be held in mind, for they make questionable the application of such mathematical treatment to some large portions of this data. It is obvious that certain influences which might affect the older trees, as explained earlier, did not affect the first-year trees. All the results with the fourth-year trees were taken in the same northern nursery during the same year. Consequently, the different trials were subject to fewer variables; this doubtless accounts for the low value of P. With a sufficiently large number of repetitions in several places and in successive years, the writers think that greater variations would probably be found. If similarly isolated groups of trials among the second- and third-year trees are taken, correspondingly low values of P are found.

Satisfactory control with adhesive tape was secured in the great majority of cases but not always. Examples may be found in table 5 in which the percentage of smooth trees from tape-wrapped grafts, while better than the controls, was only 29, 36, 42, 44, 49, 52, 58, 59, 60, etc. It appears from studies described by Riker and Hildebrand (*29*) and by Siegler and Piper (*41*) that results like these may be explained in two ways. (1) Infection of the union at the time of grafting may take place before the application of the adhesive-tape wrapper. This is perhaps pertinent when the discrepancy appears as it did once in Oklahoma on first-year trees. Such results resemble those secured from inoculations made at grafting time. (2) Infection may occur through injuries made by cultivation or by soil insects after the wrapper has decayed. This possibility has been already considered in relation to second-, third-, and fourth-year trees. This was doubtless the manner of infection on the first-year Yellow Transparent trees grown in Kansas, as recorded in table 5. Fortunately, detailed seasonal development records are available on these trees (*29*). Except for the one case in Oklahoma, these trees represent the worst failures on 1-year-old trees that the writers have experienced with tape wrappers.

The importance of infection at grafting time has been given consideration. Considerable emphasis has recently been placed on this one factor by Siegler and Piper (*41*). In an effort to discover the effect of adhesive tape upon infected unions, a number of grafts were inoculated with the hairy-root organism and then some were wrapped

with string and the others with tape. At the end of the first season, both the string-wrapped and the tape-wrapped lots showed 100 percent of hairy root. Repetitions of these trials the next year gave, respectively, 95 and 100 percent hairy root. These experiments showed that ordinary adhesive tape has little if any effect on bacteria present in the union at the time of grafting. In view of this evidence it appears that in many cases reported in tables 2 and 5 infection at the union at grafting time had comparatively little direct influence on the results. This is shown by the high percentage of smooth tape-wrapped trees at the end of the first season, suggesting that control measures based only on the importance of infection at grafting time may lead to disappointment in many cases. However, when considered from the standpoint of providing a source of inoculum for spread in the nursery by soil fauna during the latter part of the first season and during later growing seasons, as indicated by Riker and Hildebrand (*29*), it assumes more importance.

Difficulty with the adhesive tape has been encountered in three ways, none of which was serious. (1) The mechanical operation of wrapping the grafts and of cutting or tearing the tape at the proper place caused difficulty only at first. (2) Girdling of the trees during the first summer was found in about 10 percent of one planting of grafts. In this case too much tape had been wrapped about the unions, and the grafts were planted in very sandy soil. Dry weather prevailed for some time during the early part of the growing season, with the result that the tape wrapper was not sufficiently moist to decay. Even under these conditions no girdling was found on the trees that had received the right amount of wrapper. (3) A slight roughening of the bark beneath the plaster mass was noted in a few cases with certain varieties. Necrotic areas were observed which penetrated into the cortex for a short distance. Roughness of the union has sometimes been confused with the discolored residual particles of the plaster mass.

DISCUSSION

In the control of graft knots the seed used for growing seedling apple trees has been found to be of considerable importance. Since different varieties of apple trees have been shown to differ widely in their susceptibility to hairy root, it seemed probable that a similar difference in susceptibility might be found in seedlings grown from the seed of these varieties. A limited amount of evidence presented in this paper shows promise in this line of investigation. But aside from their relative resistance, which may be influenced not only by genetic constitution but also by conditions attending growth and harvesting, the seedlings may supply one or more of the other important factors.

The seedlings used for grafting may carry pathogenic bacteria. How these bacteria arrive at the surface of the seedlings is not yet perfectly understood. They may be in the soil where the seedlings are grown. However, a seemingly more important factor appears after the seedlings are dug. Ordinarily they are collected in bundles, placed in a heap, covered with packing (frequently old packing that might be classed as refuse), watered, and allowed to stand in order to "sweat off" the leaves. During this procedure it is obvious

that knot-producing bacteria on a very small percentage of infected seedlings or in the packing material might spread widely over the seedlings. There appear to be two possible remedies for this situation: (1) To prevent the spread of the bacteria; and (2) to destroy the bacteria by chemical treatments.

The bacteria carried on the surface of seedlings are aided in their entry into the union by certain cultural practices. In their effort to prevent desiccation of the seedling roots in the grafting room some nurserymen keep the roots wrapped in moist material until the minute of making the cut. Under such circumstances it has been observed repeatedly that the soil from the surface of the root may be carried over the cut surface by the knife, not only introducing any bacteria present into the union but also placing a layer of soil particles between parts of the scion and root and thus making union more difficult. Consequently, seedling roots that are clean and dry seem preferable during grafting to those that are wet and covered with soil. This appears to be quite important when the seedlings carry hairy-root bacteria, and deserves further experimental study.

The use of antiseptics on seedlings that carry hairy-root bacteria appears desirable. The determination of whether or not the infectious bacteria are carried on the surface of seedling roots is comparatively easy. By means of the technic developed by Patel (*21*) and Riker et al. (*27*), any well-equipped bacteriological laboratory might make the determinations. However, by no means all the apple seedlings carry infectious bacteria in sufficient numbers to be of primary consequence. Here again there is variation in different seedling nurseries during the same year and in the same nursery in different years. While some of the trials reported in tables 2 and 5 show that the bacteria were carried on the seedling and entered the union at grafting time, a larger number show that the union was invaded later on.

Wedge grafts appear to have no advantage over tongue grafts in the amount of graft knot developing. There was practically no difference when they were wrapped with tape. When the grafts were wrapped with string or raffia the wedge grafts had a somewhat greater tendency than tongue grafts either to come apart before planting or to send up sprouts from the root. On an average, the tongue grafts showed a slight advantage in stand.

The use of the adhesive-tape wrapper has been perhaps the most important single factor in the prevention of graft knots. Its function is rather complex. In the first place, inoculations at the union when the grafts were made indicated that the tape had little if any effect upon the entrance of bacteria at that time or on the development of infection by the bacteria that gained entrance. However, it had several other functions: (1) It prevented the further entrance of soil, water, and bacteria; (2) it reduced greatly any chance of injury to the union during various manipulations; (3) it encouraged better union between scion and root; (4) it prevented superficial development of excess callus; (5) some nurserymen have reported that it reduced the growth of mold at the union; and (6) it prevented root-chewing soil fauna from reaching the union for a number of months.

Root-chewing soil insects, including white grubs (Phyllophaga), wireworms (Elateridae), and fungus gnats (Mycetophilidae) (*29*),

may play an important role in the development of infectious hairy root not only during the first season but also during succeeding seasons. Obviously, control measures applied only at the time the grafts were made could have little effect on this factor except as they might reduce the amount of readily available inoculum. According to common entomological observation, the comparatively mild winters during the last few years probably enabled a greater number of insects to survive and have correspondingly increased their importance as a factor in the graft-knot problem. While abundant moisture in the soil favored the growth of the trees it also favored the production of callus, the activity of the insects near the surface, and the chances of entrance into the plants of any bacteria present in the soil. This serves in part to explain why graft knots are so much worse in moist than in dry years. In view of this situation, which deserves further study, such land as pasture that has been favorable to root-chewing insects seems relatively unsuited for apple grafts.

The following practices promise to contribute to the reduction of graft knots at the unions of apple trees grown from piece-root grafts:

(1) Use of apple seed from relatively resistant trees as soon as it is available.
(2) Treatment with an antiseptic of seedling roots suspected of carrying knot-producing bacteria.
(3) Use of clean dry roots when grafting.
(4) Use of a suitable wrapper, such as the adhesive tape described.
(5) Planting in soil which has been so handled that it is relatively free from root-chewing insects such as white grubs, wireworms, and fungus gnats.

SUMMARY

Graft knots of the types discussed in this paper cause severe losses in apple nursery stock propagated by piece-root grafting.

Nursery trees having infectious hairy root made, on an average, slightly less growth than smooth trees. Trees remained alive and grew slightly when all the roots but those in the hairy-root overgrowth had been removed.

The problem of controlling the overgrowths is complex, because there are several different kinds of enlargements, arising from different causes. The different kinds of overgrowths occur in various sorts and degrees of mixture.

The various overgrowths appear well distributed in the nursery. In the case of infectious hairy root, a certain amount of spread in the nursery row from hairy-root trees has been indicated.

A large percentage of the overgrowths occur at the unions. This percentage has been reduced on an average in the last 5 years, doubtless owing to improved nursery practice.

Examinations of a number of plantings have shown correlations between the length of time the trees stayed in the nursery row and the percentage of overgrowths. Although in some cases the initiation of most of the overgrowths could be traced to grafting time, in many others it was traced to the second or later seasons.

In some instances the surfaces of seedlings were found to carry hairy-root bacteria which were sources of inoculum at grafting time. In other instances this factor seemed of relatively little importance. The growing of seedling apple trees from the seed of relatively resist-

ant apple varieties gives promise of being a factor of some importance in the control of graft knots.

Antiseptic treatments of seedlings carrying bacteria that cause overgrowths seem to promise some measure of control.

Well-matched tongue grafts produced as many smooth unions as wedge grafts. Tongue grafts appeared to have certain minor advantages over wedge grafts.

Adhesive-tape wrapping appeared to be better than any other wrapping employed and to be the most important single factor among control measures. However, tape wrappers did not prevent infection at the time the grafts were made.

Control measures are discussed in relation to one another and to common nursery practice.

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TECHNIC FOR OBTAINING SPERMATOZOA FOR PHYSIOLOGICAL DAIRY STUDIES AND ARTIFICIAL INSEMINATION [1]

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INTRODUCTION

Semen may be collected from the vagina of the recently bred cow with the hand, by aspiration, or with a sponge. Semen from the vagina is satisfactory for determining whether the bull has ejaculated normal active spermatozoa during the mating, but it is unsatisfactory for use in physiological studies of spermatozoa because it is mixed with the secretions of the cow. Furthermore, collecting semen from the vagina for artificial breeding is wasteful.

A method of obtaining semen from the bull by massage of the accessory genital organs has been developed in the Bureau of Dairy Industry's physiological laboratory at Beltsville, Md. It has not been determined, however, what effect the continuous practice of obtaining semen in this way would have on the health and usefulness of the bull.

REVIEW OF THE LITERATURE

Komarov and Nagaev [2] designed a special rubber bag which they placed in the vagina of the cow. By careful technic in conducting the mating they were successful in obtaining a superior quality of semen as compared to that collected directly from the vagina with a sponge. Later, according to a report by Walton,[3] these workers used an artificial vagina and a "dummy" animal, which they claimed worked satisfactorily.

Case [4] in 1925 reported that "we procure the semen either by pressing on the seminal vesicles through the rectum, or from the vagina of the recently bred cow." In a letter to the authors in November 1932 Case described his method of massaging the seminal vesicles to obtain semen and also stated that he had used the method successfully 10 years ago in collecting semen for artificial impregnation.

ANATOMY OF THE BULL'S ACCESSORY GENITAL ORGANS

At Beltsville it was found that the seminal vesicles of the bull do not contain spermatozoa, but that the spermatozoa are in the ampullae of the ductus deferens.

The seminal vesicles and ampullae are easily identified and are so located that it is possible to manipulate the one without disturbing the other. It is more difficult to locate and manipulate the prostate of the bull because it consists of two parts and is protected by heavy muscle. The body of the prostate consists of a band which stretches across the neck of the urinary bladder and the origin of the urethra.

[1] Received for publication Mar. 16, 1934; issued July 1934.

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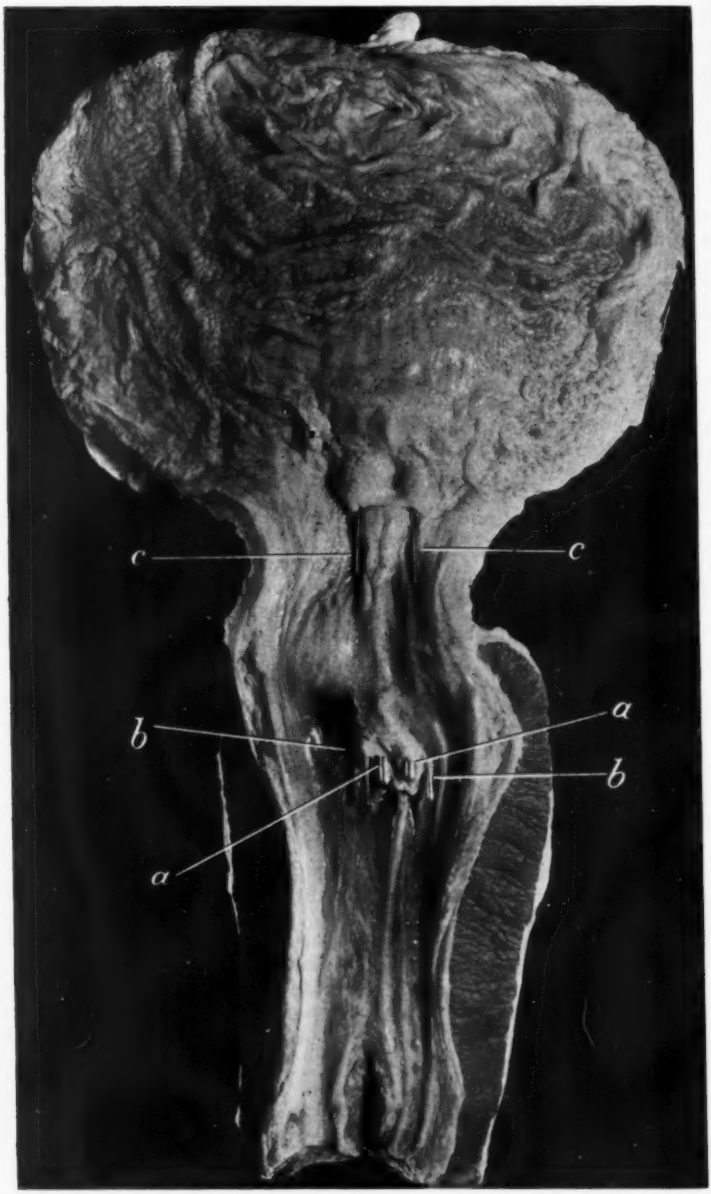

FIGURE 1.—Pelvic urethra and urinary bladder slit ventrally and laid open: *a*, Openings of ductus deferens; *b*, openings of seminal vesicles; *c*, uretral orifices.

It is about an inch and a half transversely and about half an inch in width and thickness. The pars disseminata surrounds the pelvic part of the urethra and is concealed by the urethral muscle (figs. 2 and 3).

As shown in figure 1 the ducts from the seminal vesicles and ductus deferens do not enter the urethra in a common opening; the ampullae of the ductus deferens have large lumen and the seminal vesicles have small tubules. The relation of these organs to each other is shown in figure 2.

METHOD AND RESULTS OF ITS USE

With a hand in the bull's rectum, from 7 to 10 inches, the seminal vesicles were massaged with backward strokes, and a turbid fluid flowed from the prepuce. It contained only epithelial cells in the majority of cases. In like manner the ampullae of the ductus deferens were massaged and a turbid fluid flowed out which contained only spermatozoa in the majority of cases. In some instances the ampullae were massaged first, but better results were obtained when the seminal vesicles were massaged first, because, while the ampullae were being massaged, the seminal vesicles released some of their fluid and both spermatozoa and epithelial cells were obtained. When the seminal vesicles were massaged first the epithelial cells came out, leaving only spermatozoa in the fluid obtained from the ampullae. Usually about 2 minutes of massaging gave excellent results.

In figure 3 the genital organs are shown replaced in the right half of a bull carcass. The weight of the urinary bladder has pulled the genital organs forward about an inch from the normal position in the live bull. The hand is shown massaging the seminal vesicles in figure 3, *A*, and the ampullae in figure 3, *B*.

Figure 4 shows the method of collecting the fluids as they flow out and figure 5 the comparative density of the fluids from the seminal vesicles and from the ampullae. Microscopical views of cells found in the two fluids are shown in figure 6.

Eighteen bulls ranging from 2½ to 12 years in age were used in the first 100 trials to obtain semen by massage. Epithelial cells or debris were found in 100 samples of fluid from the seminal vesicles; and in 6 of these spermatozoa were found, always from bulls that had not been used for long periods. In these six cases it is probable that the ampullae were disturbed while the seminal vesicles were being massaged.

Of the 100 trials in massaging the ampullae 81 were successful and spermatozoa were obtained from 15 bulls. Thirty-one trials were made on one bull and each time enormous quantities of spermatozoa were obtained. No spermatozoa were obtained from the ampullae of three bulls. Two of these were tried only once and the other one twice. They were disposed of before further trials were made. Failure to obtain spermatozoa was experienced in 19 trials. It was assumed that the ampullae had been emptied just previous to the time of massaging. This was indicated by the volume and tone of the ampullae, the small flaccid tube yielding no spermatozoa and the large firm tube yielding many spermatozoa.

The quantity of fluid collected from the seminal vesicles at one time varied from 0.5 to 21 cc and that from the ampullae varied from 0.5 to 23 cc.

CONCLUSIONS

Massaging the accessory genital organs of the bull is a practical way of obtaining semen for physiological studies. For artificial breed-

FIGURE 2.—Dorsal view of the internal organs of a bull: *a*, Bladder; *b*, ureter; *c*, seminal vesicles; *d*, ampullae; *e*, body of prostate; *f*, pelvic urethra; *g*, bulbo-urethral (Cowper's) glands.

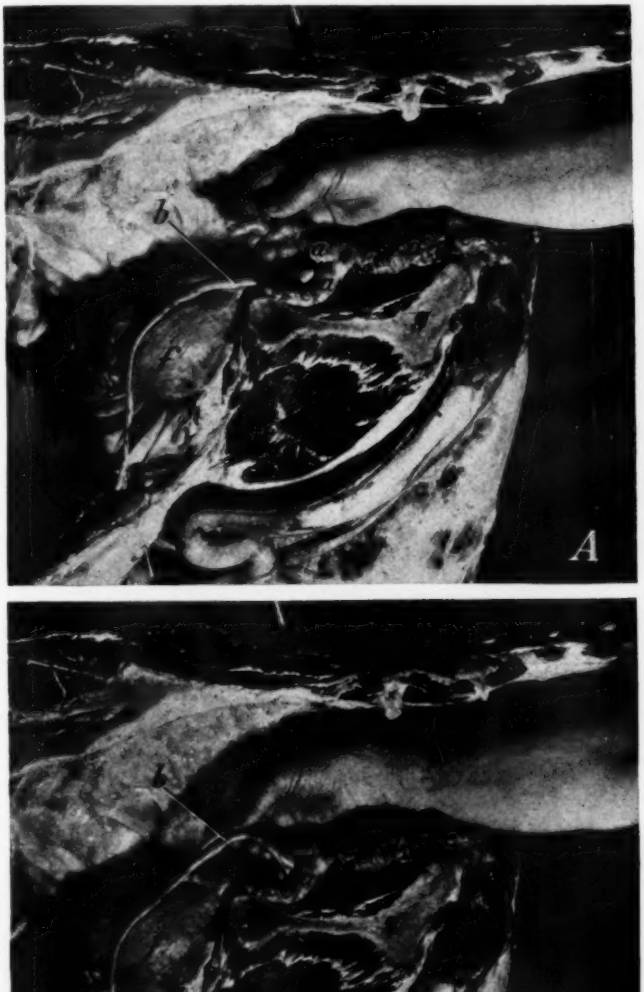

FIGURE 3.—Position of the genital organs of the bull and method of manipulating them: *A*, Massaging the seminal vesicles; *B*, massaging the ampullae of the ductus deferens. The organs are: *a*, Seminal vesicles; *b*, ampullae; *c*, body of prostate; *d*, pelvic urethra; *e*, bulbo-urethral (Cowper's) glands; *f*, urinary bladder; *g*, pubis

FIGURE 4.—Method of collecting semen with funnel and test tube.

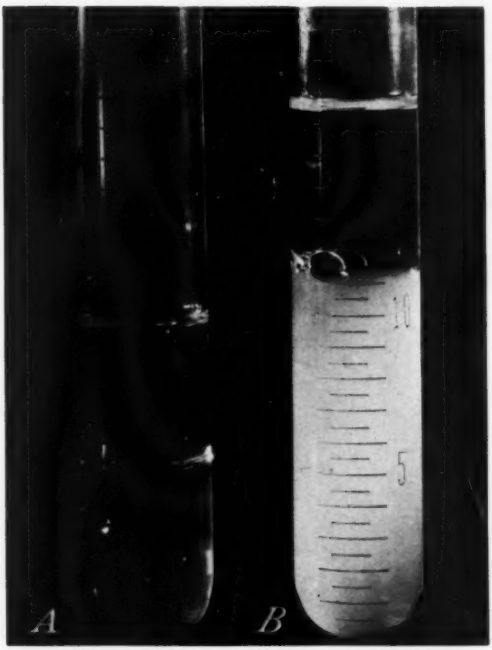

FIGURE 5.—Material collected from (*A*) seminal vesicles and (*B*) ampullae.

ing purposes it is desirable to massage the ampullae, for from this organ will be obtained the greatest volume of semen containing active spermatozoa. The method is also useful with valuable breeding bulls

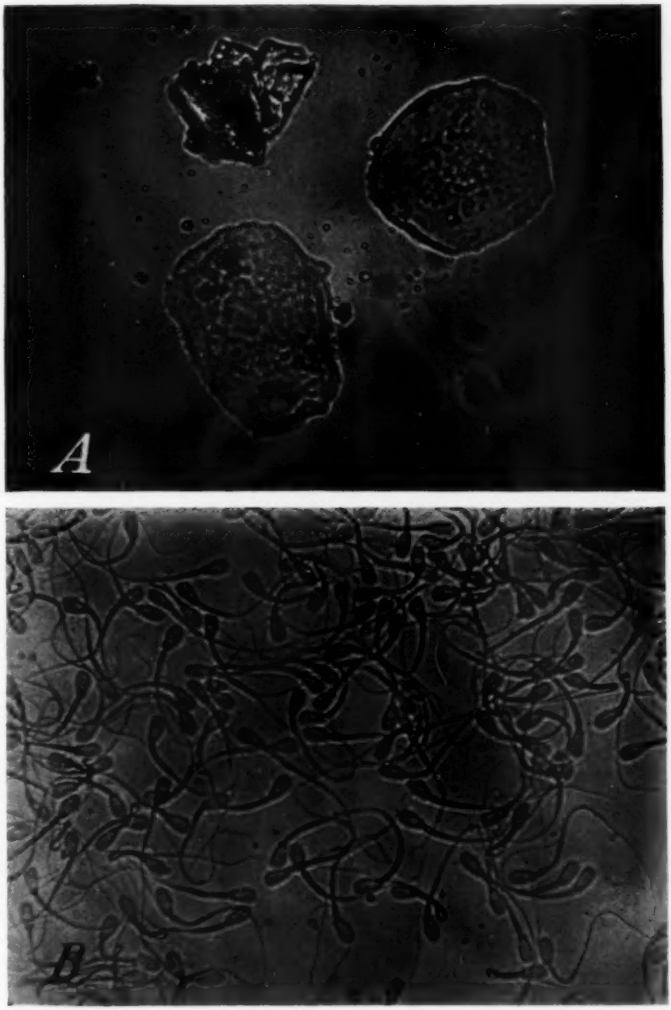

FIGURE 6.—*A*, Epithelial cells from seminal vesicles; *B*, spermatozoa from ampullae. × 450.

that are unable to serve cows in the normal manner because of injury. Other advantages of collecting semen directly from the bull for use in artificial breeding are that it prevents waste of semen and produces semen free from extraneous matter.

APHELENCHOIDES XYLOPHILUS, N. SP., A NEMATODE ASSOCIATED WITH BLUE-STAIN AND OTHER FUNGI IN TIMBER [1]

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INTRODUCTION

A new case of unusual ecological conditions to which nematodes have adapted themselves has been found in a nematode species apparently specialized to live in timber affected by blue-stain and other wood fungi. This new species is described herein.

ECOLOGICAL RELATIONS

The first observation of *Aphelenchoides xylophilus*, n.sp., the timber nema, dates back to 1929, when a small piece of wood that had been cut, in the process of roofing, from the top of a green pole of longleaf Louisiana pine (*Pinus palustris* Mill.) was received from Orange, Tex.[2] This piece of wood had streaks of a bluish color caused by blue-stain fungi. The nemas were found in these streaks and in bordering portions. Larval specimens, males and females, were observed. Although they were not numerous, a dozen or more specimens could be found in a small portion of wood when soaked and dissected properly. Soaking the wood in water activated the nematodes, whereas drying the wood induced dormancy. Some tests showed revival of the nematodes after a dormancy of 1 year but not after 2 years.

Later, through the courtesy of Ross W. Davidson, of the Division of Forest Pathology, Bureau of Plant Industry, there were received four different plate cultures of wood fungi in which nematodes had developed. All of these nematodes proved to be *Aphelenchoides xylophilus*. Three of the cultures were from a sawmill in Bogalusa, La., and were also obtained from blue-stained logs of *Pinus palustris*. These logs had previously been attacked by beetles of the genus *Ips*, which, according to Davidson, usually carry the blue-stain fungus *Ceratostomella ips* Rumbold, but which in these three cases contained a brown fungus belonging probably to the genus *Trichosporium*, of the "Fungi Imperfecti."

The fourth culture on which the same species of nematode developed was obtained from a pine tree (*Pinus echinata* Mill.) that had been recently killed by an attack of the beetle, *Dendroctonus frontalis* Zimm., near Fairfax, Va. In this case the nematodes originated in the interior of unstained wood, one-sixteenth to one-fourth of an inch below the insect galleries. The fungus here associated with this nematode is said by Davidson to be entirely hyaline and also to belong

[1] Received for publication Apr. 4, 1934; issued July 1934.

[2] Received through the courtesy of T. E. Snyder, of the Bureau of Entomology, U.S. Department of Agriculture, who received the wood from C. H. Lyon, chemist of the Texas Creosoting Co. Mr. Lyon wrote: "All such poles came from an area including western Louisiana and southeastern Texas. The climate is hot and, at the time of finding that specimen, was very humid. The average annual humidity is given by the Government observer of this region as of 83.3 percent. The specimen had not been treated nor come in contact with creosote. It has been at all times exposed to weather."

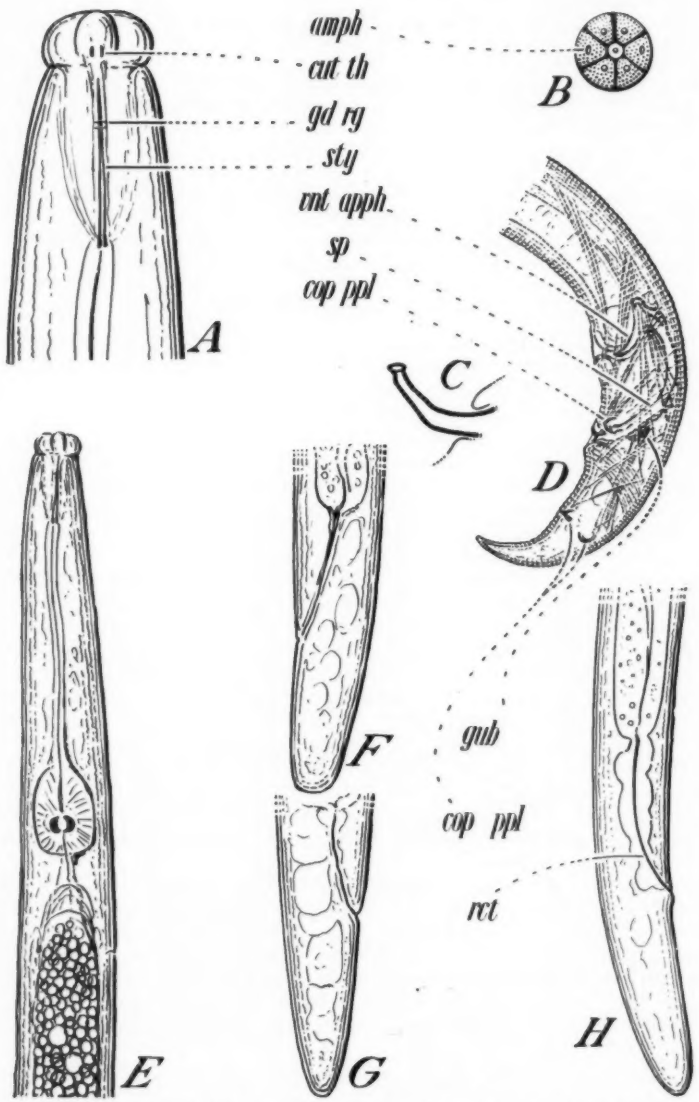

FIGURE 1.—*Aphelenchoides xylophilus*, n.sp. *A*.—Head of female: *cut th*, Cuticular thickening in cephalic portion of alimentary tract; *gd rg*, guiding rings of stylet; *sty*, stylet. × 2,800. *B*.—Front view of head: *amph*, Amphid. × 1,370. *C*.—Extruded spicula showing circular expansion. × 1,370. *D*.—Tail of male: *vnt apph*, Ventral apophysis; *sp*, spicula; *cop ppl*, copulatory papillae (three pairs); *gub*, gubernaculum. × 1,060. *E*.—Anterior end of larva. × 1,060. *F* and *G*.—Tails of larvae, showing variation in shape. × 1,060. *H*.—Tail of female: *rct*, Rectum. × 1,060.

to the "Fungi Imperfecti." However, according to the same authority, this particular "isolation" was taken only a short distance (1 inch or less) from wood which was blue-stained by *Ceratostomella pini* Münch, a fungus associated with *D. frontalis*. It is thought that the nematode might also have been present in this blue-stained wood.

This constant association suggests that the nematode described herein uses the insects as carriers and probably feeds on the various fungi involved in the same association. Similar carrier relationships between nematodes and insects are known, especially in connection with bark beetles, dung beetles, flies frequenting fermenting substances, etc. In this respect the observations made herein offer nothing new, but the apparent specialization of this nematode to a life in wood and its association with fungi of the blue-stain type merit special attention.

TECHNICAL DESCRIPTION

Aphelenchoides xylophilus, n.sp.

Like the other members of the genus, *Aphelenchoides xylophilus* is of slender shape and has the following dimensions:

♀	1.8	7.9	8.7	74.	96.2	0.9 mm.
	1.0	1.5	1.5	1.6	1.0	
♂	2.2	9.3	10.	M	95.9	0.77 mm.
	1.2	1.6	1.6	2.0	1.5	

The cuticle is very finely annulated (8 annules to 6μ in the head region); the head well set off; the tail of the larva and female more or less obtuse and slightly longer than the rectum (fig. 1, *F*, *G*, *H*), that of the male conically pointed, ventrally curved, and slightly longer than the spiculum (fig. 1, *D*). A front view of the head shows a six-radiate cuticular structure. The lobes between the radii carry the sense organs in the order typical for the genus, i.e., on the lateral lobe the amphids, on each submedial lobe one papilla (fig. 1, *B*). The stylet is very fine and about one and one-half times as long as the head is wide; its knobs are minute; two fine guiding rings are present and the wall of the cephalic portion of the alimentary tract is reinforced by short cuticular thickenings (fig. 1, *A*). It seems that the esophageal glands open in the middle bulb of the esophagus in the manner typical of the genus; the bodies of the glands, however, have a dorsal situation outside the alimentary tract at the beginning of the intestine; they extend to about 85μ behind the esophageal bulb and have a strictly serial arrangement. The length of the rectum is about twice the anal body diameter. An obscure excretory pore opens ventrad of the nerve ring (fig. 1, *E*). The vulva is a narrow transverse slit but stands out rather well because the body narrows suddenly behind it. The testis is outstretched forward to the right of the intestine, and ends about 300μ behind the esophageal bulb. The spicula resemble those of other members of the genus but have in addition an extremely well-developed ventral apophysis at the proximal end. In some specimens this apophysis seemed to connect with the ventral body wall (fig. 1, *D*), but in others no such connection was seen. Figure 1, *C*, shows the distal end of the spiculum as forming a circular expansion. A small gubernaculum is present. The copulatory musculature is shown in figure 1, *D*. There are two pairs of large, somewhat mammillate ventrosubmedial copulatory papillae (fig. 1, *D*), one pair at about the middle of the tail and the other just in front of the anus. A third pair seems to have a dorsolaterad position also in the middle of the tail.

DIAGNOSIS.—*Aphelenchoides* with an obtusely rounded conical tail in the larva and in the female, but pointed in the male, with a fine, barely knobbed buccal stylet. The spiculum of the male proximally with long ventral apophysis; a short, lineate gubernaculum present; male tail with large mammillate copulatory papillae; a pair ventrosubmedial in front of anus, a second pair ventrosubmedial in the middle of the tail, and a third dorsolaterad also in the middle of the tail. Associated with blue-stain and similar wood fungi.

TYPE HOST.—*Pinus palustris*.

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